Biochimica et Biophysica Acta 1787 (2009) 747–752

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Properties of zeaxanthin and its radical cation bound to the minor light-harvesting complexes CP24, CP26 and CP29

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ARTICLE INFO

Article history:
Received 19 December 2008
Received in revised form 12 February 2009
Accepted 12 February 2009
Available online 24 February 2009

Keywords:
Nonphotochemical quenching
Ultrafast spectroscopy
Photoprotection
Minor light harvesting complexes
Beta-carotene

ABSTRACT

Nonphotochemical quenching (NPQ) is a fundamental mechanism in photosynthesis by which plants protect themselves against excess excitation energy and which is thus of crucial importance for plant survival and fitness. Recently, carotenoid radical cation ($Car^{\bullet+}$) formation has been discovered to be a key step in the feedback deexcitation quenching component (qE) of NPQ, whose molecular mechanism and location remains elusive. A recent model for qE suggests that the replacement of violaxanthin (Vio) by zeaxanthin (Zea) in photosynthetic pigment binding pockets can in principle result in qE via the so-called "gear-shift" or electron transfer quenching mechanisms. We performed pump-probe measurements on individual antenna complexes of photosystem II (CP24, CP26 and CP29) upon excitation of the chlorophylls (Chl) into their first excited Q_y state at 660 nm when either Vio or Zea was bound to those complexes. The Chl lifetime was then probed by measuring the decay kinetics of the Chl excited state absorption (ESA) at 900 nm. The charge-transfer quenching mechanism, which is characterized by a spectral signature of the transiently formed Zea radical cation ($Zea^{\bullet+}$) in the near-IR, has also been addressed, both in solution and in light-harvesting complexes of photosystem II (LHC-II). Applying resonant two-photon two-color ionization (R2P2CI) spectroscopy we show here the formation of β-$Car^{\bullet+}$ in solution, which occurs on a femtosecond time-scale by direct electron transfer to the solvent. The β-$Car^{\bullet+}$ maxima strongly depend on the solvent polarity. Moreover, our two-color two-photon spectroscopy on CP29 reveals the spectral position of $Zea^{\bullet+}$ in the near-IR region at 980 nm.

1. Introduction

Feedback deexcitation (qE), the high-energy dependent component of NPQ is an important process in the photoprotection of plants during which the light harvesting antennae switch to specific states to dissipate excitation energy of chlorophylls (Chls) as heat [1–4]. Along with other factors like pH change [5], activation of qE is correlated with zeaxanthin (Zea) formation [5,6]. Although the interconversion of violaxanthin (Vio) to Zea in the xanthophyll cycle is a prerequisite for complete qE induction, the detailed molecular mechanism of qE remains elusive. Today even the exact role of carotenoids in this process is unclear [7–9].

According to the so-called "gear-shift" model [7], Zea acts as a direct quencher of electronically excited Chls, since its first singlet excited electronic state (S_1) is supposed to be lower than the lowest excited Chl state (Q_y). Excitation energy transfer (EET) from Chl *a* to Zea is thus in principle possible and energy dissipation at Zea can take place [7,10]. In contrast, the S_1 state of Vio is supposed to be above the Q_y level of Chl *a* and thus cannot quench the Chl *a* excited state. This mechanism has been recently verified in artificial light-harvesting dyads [11]. Still, it remains to be shown if this mechanism applies also to natural systems.

In addition, it is thought that Zea forms a quenching complex with Chl *a* during the induction time of qE, and Zea acts as terminal quencher via electron transfer, which leads to carotenoid radical cation formation [9,12–16]. This hypothesis was first predicted by theory [14–16], and has been confirmed experimentally [9]. The corresponding experiment was performed on intact spinach thylakoid membranes and the near-IR region was probed after excitation at 664 nm under quenched and unquenched conditions. The spectral differences observed between the quenched and unquenched states were ascribed to the formation of Zea radical cations ($Zea^{\bullet+}$). The charge transfer state is formed from relaxation of the Chl–Zea excited state, (Chl–Zea)* [9]. Transient generation of $Zea^{\bullet+}$ in ~11 ps was observed, which corresponds to the net dynamics of the Chl bulk molecules that transfer the excitation energy to the Chl–Zea heterodimer, followed by an ultrafast charge separation leading to a $Chl^{-\bullet}$ and a $Zea^{\bullet+}$. The charge separated $Zea^{\bullet+}$ can be detected via a transient absorption signal in the spectral region specific for carotenoid radical cations. Subsequently the $Zea^{\bullet+}$ signal decays on a timescale of 150 ps, which corresponds to charge recombination between $Chl^{-\bullet}$ and $Zea^{\bullet+}$.

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doi:10.1016/j.bbabio.2009.02.006

Recent studies in which the $Zea^{\bullet+}$ signature has been used to infer qE quenching suggest that the minor light-harvesting complexes (minor LHCs or mLHCs) (CP24, CP26 and CP29) provide potential sites for this observed electron transfer quenching [12,13]. Nevertheless, it remains to be shown, where exactly and in which pigment–protein complex of the photosynthetic apparatus qE actually occurs.

Currently, there are a few studies addressing the properties of carotenoid radical cations ($Car^{\bullet+}$) and anions ($Car^{\bullet-}$) directly [17–19]. Although the β-carotene radical cation (β-$Car^{\bullet+}$) in reaction centers has been studied for over 20 years [20,21], spectroscopic investigations of such cations generated electrochemically in solution have been reported only recently [22,23]. $Car^{\bullet+}$ have also been observed in LH2 from purple bacteria upon direct carotenoid excitation [24].

Recently, we have generated and characterized $Car^{\bullet+}$ by means of resonant two-photon two-color ionization (R2P2CI) spectroscopy both in solution and in light harvesting complexes of photosystem II (LHC-II) [25]. However, despite their fundamental importance in NPQ of green plants, only little is known about the $Car^{\bullet+}$ excited states and their dynamics. Our recent detailed studies of isolated carotenids in solution reveal the excited state dynamics of $Car^{\bullet+}$ [26]. Our findings have direct implications for NPQ, since direct quenching of Chl excited states by Förster energy transfer to $Car^{\bullet+}$ should be possible and efficient [23].

In this paper, we use multiple light pulses to investigate the ultrafast dynamics of $Car^{\bullet+}$ formation both in solution and in plant mLHCs. The three-pulse technique comprises two high-intensity pulses initiating a R2P2CI process and a low-intensity broad-band probe pulse. The R2P2CI measurements reveal the spectroscopic properties of β-$Car^{\bullet+}$ in solution and the spectral characteristics of $Zea^{\bullet+}$ in CP29.

2. Experimental methods

β-Car was purchased from Sigma and stored at −20 °C. Recombinant CP24, CP26 and CP29 from *Arabidopsis thaliana* were refolded in the presence of isolated pigments with adjusted chlorophyll content in order to resemble native mLHCs [27]. Incubation of CP24, CP26 and CP29 with Zea yielded Zea-containing mLHCs (CP24-Zea, CP26-Zea and CP29-Zea). Pigment composition was determined by HPLC analysis. The typical sample OD was 0.4 0.8/mm at 665 nm. Sample stability was confirmed by measuring the absorption spectra before and after the time resolved measurements.

The time resolved measurements were performed using a CLARK CPA 2001 (Dexter, MI) laser/amplifier system operating at a repetition rate of ~1 kHz at a central wavelength of 775 nm. The recombinant CP24, CP26 and CP29 were excited by pulses generated using a noncollinear optical parametric amplifier (NOPA) with the output tuned to 660 nm for which the maximum excitation energy was kept around 10 nJ.

For the R2P2CI process the amplified pulses were divided into three parts: two pump pulses and one probe pulse. The first excitation pulse was generated using a NOPA and the maximum excitation energy was kept at 60 nJ for a central wavelength of 490 nm. The second pump pulse, with an energy of 200 nJ at a central wavelength of 775 nm, was delayed by 40 fs with respect to the first pulse. For the probe pulses a white light continuum was generated by focusing amplified 775 nm light into a 5 mm sapphire window and a RG 830 filter was used to select the desired spectral region. For further experimental details see [25].

3. Results

3.1. Chlorophyll excited-state dynamics in mLHC proteins

3.1.1. Excitation energy transfer

Several models for qE have been proposed and are still discussed controversially [8,15,28,29]. A recent model for qE suggests that the replacement of Vio by Zea in its LHC-II binding pocket can in principle result in qE via the so-called "gear-shift" mechanism which requires the Vio S_1 state to be higher in energy than the Q_y of Chl *a*, and the S_1 state of Zea to be lower [3]. Pump-probe experiments on isolated Zea enriched LHC-II complexes, as well as theoretical calculations have proven that this replacement alone is not sufficient. However, this appealingly simple mechanism could still be possible for minor LHCs CP24, CP26 and CP29, which all bind Vio. However, the exact molecular structure of these complexes is unknown. To test the proposed model, ultrafast pump-probe measurements were performed on mLHC–Vio and mLHC–Zea complexes. The experimental approach employs excitation of mLHCs chlorophylls at 660 nm into the first excited state and measurement of the lifetime of Chl *a* via the decay kinetics of the excited state absorption (ESA) of the Chl Q_y excited state at 900 nm. According to the "gear-shift" model and calculations on Car–Chl model complexes [30], EET from Chl(Q_y) to Vio(S_1) should not be possible (left side of Fig. 1), while excitation energy located on a Chl may be transferred to Zea(S_1) (right side of Fig. 1), which finally decays non-radiatively by internal conversion (IC) back to the electronic ground state.

The presence of Zea in the refolded minor light-harvesting complexes (CP24, CP26 and CP29) was verified by HPLC analysis (Table 1). In the mLHC–Vio samples no trace of Zea was detected, while the Zea-enriched mLHC samples (CP24-Zea, CP26-Zea and CP29-Zea) contain a significant amount of Zea. The Lut content remains virtually constant in the CP24 samples, while no trace of neoxanthin (Neo) was detected for both CP24 samples. The amount of Vio decreases in the CP24-Zea compared to CP24-Vio, indicating Vio to Zea exchange. The amount of Zea in CP26-Zea reaches about the same level as Lut or Neo, while the Vio content of CP26 and CP26-Zea is low. For the CP26 samples, a significant decrease of the Lut content from CP26-Vio to CP26-Zea is observed, thus Lut appears to be replaced by Zea. Also, it should be noted that a part of Vio is lost during the pigment modification procedure for the CP24 and CP26 but not for CP29. For both CP29 samples, the levels of Lut, Neo and Vio remain virtually constant, while Zea seems to be simply added and not replacing a specific native carotenoid, or it may bind to empty pockets. The data in Table 1 are normalized to equal amounts of Chl in mLHC–Vio and mLHC–Zea.

To study the kinetics of the first excited Chl state in mLHC, the Q_y transition was excited at 660 nm and transient absorption (TA) spectra were acquired in the near-IR region from 800 to 1050 nm, where the broad ESA of Chl can be detected. The near-IR TA kinetic profiles at 900 nm (Fig. 2) originate solely from Chl ESA dynamics [9,25,31], which decay with two time-constants. The TA spectra were analyzed using a global fitting routine, revealing two major decay components, a fast decay component with lifetimes of 3.8, 4.2 and 5 ps for CP24, CP26 and CP29 respectively, and a slow component which extends beyond the time scale of our measurements: 2.9 ns for CP24, 2.35 ns for CP26 and 2.2 ns for CP29. Comparing the TA measurements of all mLHC samples we found kinetic differences between individual

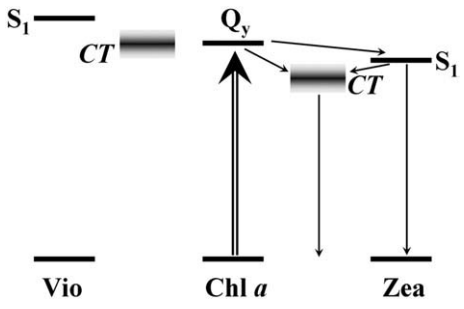

Fig. 1. Schematic representation of the extended gear-shift model. The S_1 level of Vio, Zea and the CT energy levels are calculated by Wormit et al. [39]. Locally excited state of Vio, Zea and Chl *a* are represented by bars, while charge transfer states (CT) are represented by energy boxes accordingly to their distance dependence character.

Table 1
HPLC analysis for mLHC–Vio and mLHC–Zea. The data are normalized to equal amounts of Chl

	LUT	NEO	VIO	ZEA	Chl *b*	Chl *a*
CP24-Vio	0.92	0	1.1	0	5.75	4.23
CP24-Zea	0.82	0	.9	0.38	5.78	4.20
CP26-Vio	1.35	0.58	.18	0	3	6
CP26-Zea	0.85	0.67	.1	0.5	3.09	5.91
CP29-Vio	0.91	0.51	0.53	0	2.01	5.98
CP29-Zea	0.85	0.49	0.51	0.24	2.27	5.73

complexes, i.e. different amplitudes and lifetimes of the fast component. On the other hand, the TA measurements of the Zea and Vio enriched samples of the same complex, e.g. CP24-Zea and CP24-Vio, are virtually identical (Fig. 2). These data are consistent with the results obtained for LHC-II binding Vio or Zea [25].

3.2. Generation of carotenoid radical cations in solution

As proof of principle and to demonstrate the usefulness of our spectroscopic approach, we first discuss details of the generation of $Car^{\bullet+}$ by R2P2CI in solution and describe results obtained for β-Car in increasingly polarizability solvents. Excitation of carotenoids with a pump pulse centered at 490 nm promotes the carotenoids into the S_2 excited state. Applying a second pulse (775 nm), while the S_2 state is occupied results in a further excitation of the carotenoid molecules into an autoionizing S_N state. This higher excited state can either relax back to S_2 or an electron can be released to solvent resulting in a solvated electron, and the formation of a $Car^{\bullet+}$ (Fig. 3B). The second

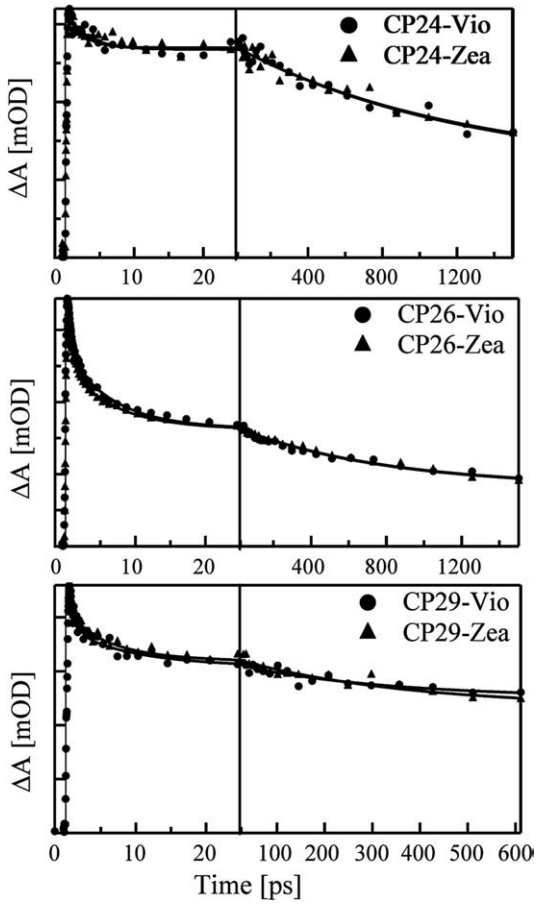

Fig. 2. Transient absorption data for mLHC–Vio (circles) and mLHC–Zea (triangles) detected at 904 nm (ESA of Chl *a*) upon excitation at 660 nm. The solid lines correspond to fit curves obtained by a global fitting routine.

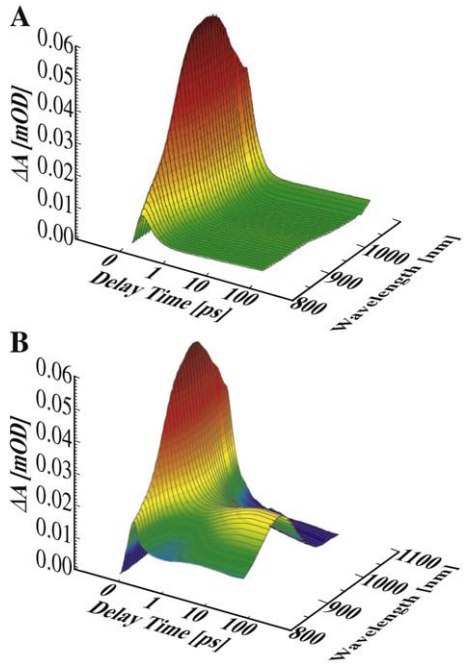

Fig. 3. Temporal evolution of β-Car upon excitation at 490 nm (A) and of $β\text{-}Car^{\bullet+}$ upon generation by R2P2CI (B).

pathway is supported by previous studies [25], and leads to a characteristic spectral signature of $Car^{\bullet+}$ in the near-IR region. In a typical example, Fig. 3 shows pump-probe (PP) (*A*) and R2P2CI (*B*) measurements performed on β-Car in chloroform. The photoinduced absorption originating from the $Car^{\bullet+}$ (Fig. 3B) is clearly separated from the strong excited state absorption of the $S_2 \rightarrow S_N$ transition by their sequential occurrence; the first has a lifetime on the order of μs while the latter is very short lived, with $\tau \approx 100$ fs.

TA spectra of $β\text{-}Car^{\bullet+}$ generated as above in decreasingly polar solvents (acetonitrile, ethanol, acetone, dichloromethane, chloroform and CS_2) are presented in Fig. 4. The spectra are taken at a delay time of 50 ps. For easy comparison, the $β\text{-}Car^{\bullet+}$ spectra are normalized to the same maximum absorbance. The $β\text{-}Car^{\bullet+}$ absorption band shows a strong solvent dependent spectral change; its λ_{max} shifts to lower energy upon increasing polarizability, i.e. from acetonitrile to CS_2 by about 130 nm, reflecting the carotenoid-to-solvent dispersive interaction (Fig. 4).

3.3. Carotenoid radical cation detection in CP29

R2P2CI measurements on LHC-II indicated a chlorophyll ESA signal as a consequence of EET from S_2 (Car) to Q_y (Chl) which interferes

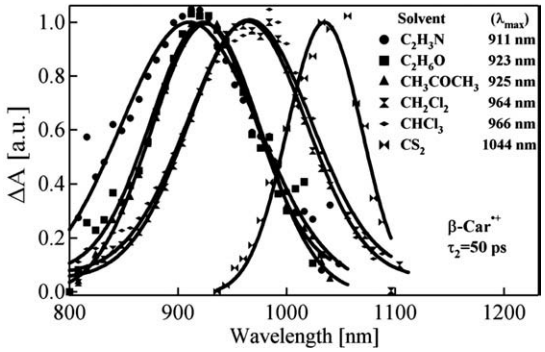

Fig. 4. Transient absorption spectra of $β\text{-}Car^{\bullet+}$ in acetonitrile, ethanol, acetone, dichloromethane, chloroform and carbon disulfide. τ_2 represents the delay time between the first pulse and the probing pulse.

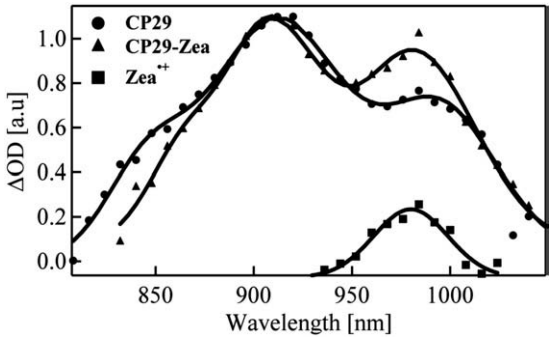

Fig. 5. Difference transient absorption spectra between R2P2CI and PP revealing all $Car^{\bullet+}$ in CP29 (circles) and CP29-Zea (triangles). The solid line corresponds to Gaussian fits of the data points. The difference between CP29-Zea and CP29 (squares) and the respective Gaussian fits with a maximum at 980 nm reflects the $Zea^{\bullet+}$ band.

with the detection of $Car^{\bullet+}$ [25]. Accordingly, the PP and R2P2CI spectra of CP29-Vio and CP29-Zea recorded at a delay of 40 ps upon carotenoid excitation into S_2 at 490 nm are dominated by a structureless ESA signal, which originates from excited Chls and cover the entire investigated spectral range. However, after 40 ps all the excitation energy is already transferred to Chls as a result of direct EET from the carotenoids. Thus, the PP spectrum at 40 ps contains exclusively Chl ESA, while a corresponding R2P2CI spectrum contains both Chl ESA and $Car^{\bullet+}$ contributions. The difference between R2P2CI and PP spectrum at 40 ps should thus reflect only the remaining spectral contribution of the thermalized $Car^{\bullet+}$ species.

Application of a R2P2CI pulse sequence to the CP29-Vio and CP29-Zea samples lead to the spectroscopic generation of a transient ESA signal which corresponds to the sum of all different carotenoid cations present in CP29-Vio (Lut, Neo and Vio) or CP29-Zea (Lut, Neo, Vio and Zea). Fig. 5 shows the transient absorption spectra recorded at 40 ps time delay following two-photon two-color excitation of CP29-Vio (circles) and CP29-Zea (squares). The positive component of the difference transient absorption spectrum CP29-Zea CP29-Vio (triangles) corresponds to the $Zea^{\bullet+}$ band. A Gaussian fit of the data points yields a maximum at 980 nm. The peak at 910 nm which shows the same amplitude for both CP29-Vio and CP29-Zea samples can be attributed to $Vio^{\bullet+}$ in analogy with our previous study on LHC II [25]. According to the same study the $Lut^{\bullet+}$ band will mainly absorb in the region between 950 and 1000 nm. A recent study of chemically generated $Neo^{\bullet+}$ and $Vio^{\bullet+}$ performed in dichloromethane [32], reveals the cationic bands at 852 nm and 856 nm, respectively. Assuming that both cations will maintain the same spectral sift in the protein environment, the $Neo^{\bullet+}$ cation band will thus contribute to the peak around 910 nm. Neoxanthin is reported not to be involved directly in the quenching process [8], however, it may be relevant for protein stability.

4. Discussion

The global fit analysis of the TA measurements on all mLHC samples performed over the entire investigated spectral range (800–1050 nm) upon Q_y excitation revealed two kinetic components, a fast 3.8–5 ps and a slow 2.2–2.9 ns component. The kinetics observed within the same type of mLHC samples are essentially identical over the entire investigated spectral range, displaying only minor differences, which are not significant within our experimental errors. The transient characteristics in the Q_y region are illustrated by the ESA dynamics at a probe wavelength of 900 nm (Fig. 2). Earlier time-resolved multicolor pump-probe measurements reveal the energy transfer process within the minor light-harvesting antenna complex CP29 of green plants [33]. In this study, the energy flow from the Chl *b* to Chl *a* molecules reveal time constants of 350 fs (from the "blue" Chl *b* to the "red" Chl *a*) and 2.2 ps (from the 'red' Chl *b* to the "blue" Chl *a*). Furthermore, fast 280 fs and slow 10–13 ps equilibration processes among the Chl *a* molecules were observed. In agreement with these earlier measurements we assign our first component to the equilibration between Chl *a* molecules and EET from Chl *b* to Chl *a*, while the slow decay component is attributed to the excited state lifetime of Chl *a* molecules.

Mullineaux et al. showed that the Zea/Vio ratio in LHC-II has no influence on the time-resolved fluorescence emission spectrum [34]. However, the authors report a decrease of the fluorescence lifetime from 4.3 ns in detergent-solubilized LHC-II to 110 ps in a semicrystalline aggregated state. They also state that the quenching species is a nonfluorescent pigment, which cannot be resolved by time-resolved fluorescence measurements. This difficulty is overcome by our measurements since here we measure ESA and can thus detect also nonfluorescent (dark) states. Our experiments reveal no change in lifetime of the excited state absorption signal of the initially excited Q_y states. Both the mLHC–Vio and mLHC–Zea samples exhibit the same excited state dynamics and no evidence of Chl excited state quenching has been found. Since the Chl fluorescence lifetime is typically 2.5 ns, it is unlikely that relevant changes occur beyond the time range of our experiments of 1.5 ns.

In a previous paper [25], we demonstrated clearly that the replacement of Vio by Zea in the major light-harvesting complex II (LHC-II) has no influence on the Chl excited state lifetime. The same experimental approach applied here on refolded minor light-harvesting complexes, which in the photosynthetic system occupy an intermediate position between the peripheral LHC-II and the reaction center, support the conclusion that the presence of Zea does not lead to Chl quenching by EET. In this view, the absence of EET from the lowest Chl excited state to the lowest Zea excited state may be explained in terms of weak coupling of these two excited states, due to the small transition dipole moment of the carotenoid S_1 excited state.

The other NPQ model discussed in the introduction, in which Zea is thought to form a quenching complex with Chl *a*, has provide experimental and theoretical evidence of charge transfer quenching through formation of $Car^{\bullet+}$. This hypothesis was first predicted by theory [14], showing that for a suitable geometry of the Chl–Zea heterodimer quenching by charge transfer is possible; this corresponds to non-radiative decay into an appropriate energetically low-lying charge transfer excited state [15]. The corresponding experiment was performed on intact spinach thylakoid membranes and the near-IR region was probed after excitation at 664 nm under quenched and unquenched conditions [9]. The spectral differences observed between the quenched and unquenched states were ascribed to the formation of $Zea^{\bullet+}$ based on *A. thaliana* mutants with distinct carotenoid composition. Thus, the necessity of Zea for the generation of the kinetic changes allows the specific assignment of the species observed during qE as a $Zea^{\bullet+}$. Recent studies in which the $Zea^{\bullet+}$ signature has been used to infer qE quenching suggest that all mLHCs (CP24, CP26 and CP29) provide potential sites for charge transfer quenching [12,13]. In our PP study only CP26-Zea, with a significant Zea content shows hints for a $Car^{\bullet+}$, though this signal lies within the detection errors and thus we will not discuss it further. All other difference spectra do not show the absorption signal expected for $Zea^{\bullet+}$. This might be attributed to a lower amount of Zea incorporated in our mLHCs. In our experimental approach, the pigment exchange procedure is applied to refolded mLHCs that carry a native-like carotenoid content. On the other hand, the mLHCs used by Ahn et al. were refolded with pigment mixtures only containing Lut and either Vio or Zea [12]. Nevertheless, the signal observed for the mLHCs [13], is about 50 times smaller and 20 nm blue-shifted, compared to what has been observed in intact thylakoids [9]. Considering this, the spectral properties and position of $Zea^{\bullet+}$ in mLHCs still remain to be clarified. We thus performed R2P2CI measurements to investigate the ultrafast dynamics of $Car^{\bullet+}$ formation in CP29 and to reveal the spectroscopic signature of $Zea^{\bullet+}$ in the Zea-enriched CP29.

The temporal and spectral evolution of the optically generated β-Car$^{\bullet+}$ species is presented in Fig. 3. In the R2P2CI experiments a long-lived species with a TA signal in the near-IR region was observed for β-Car in all investigated solutions. In previous experiments, transient Car$^{\bullet+}$ species were created by ionization, which vanish only after charge recombination, a process which occurs typically within ps to μs [35]. Previously reported pump deplete probe (PDP) experiments on lycopene showed a persistent loss of excited-state population after re-excitation of the S_2 ESA at 795 nm [36]. No product state was observed, and the re-pumped population disappeared, most probably deposited in a long-lived Car$^{\bullet+}$ state with no clear spectral signature in the probed region. Similarly, double-pump techniques have been applied to peridinin, where an 800 nm pulse was applied soon after carotenoid excitation. Thereby, a part of the S_2 population was transferred to a higher excited state, resulting in a photoproduct state persisting on a nanosecond time scale, that was attributed to peridinin cations [37]. The R2P2CI experiments performed on β-Car show a similar transient species as reported previously [25], which can unambiguously be assigned to β-Car$^{\bullet+}$.

In general, Car$^{\bullet+}$ absorption strongly depends on the solvent as previous chemical oxidation studies illustrate. For example, β-Car$^{\bullet+}$ absorbs at 935 nm in acetonitrile [22], and 970 nm in dichloromethane [38]. In our R2P2CI study, the general position of the β-Car$^{\bullet+}$ absorption band is determined by the polarizability of the solvent. Thus β-Car$^{\bullet+}$ shows maxima at 911 nm in acetonitrile, at 923 nm in ethanol, at 925 nm in acetone, at 964 nm in dichloromethane, at 966 nm in chloroform and at 1044 nm in CS_2.

The absorption maxima of β-Car$^{\bullet+}$ generated by chemical oxidation display the same tendency with respect to the solvent polarizability, though the spectral shift is less pronounced [26]. Since the formation of β-Car$^{\bullet+}$ upon R2P2CI occurs on a femtosecond time-scale by direct electron ejection into the solvent, the observed difference between R2P2CI and chemical oxidation is not surprising. In these terms, the ejected electron provides a major environmental change that has a strong effect on the optical properties of β-Car$^{\bullet+}$.

The main goal of the present work was to investigate and characterize Zea$^{\bullet+}$ in CP29 and to understand its role in qE. Fig. 5 presents the R2P2CI-PP difference spectra of CP29 and CP29-Zea samples which correspond to sums of all different carotenoid cations spectroscopically generated in the R2P2CI experiment (Vio, neoxanthin (Neo), lutein (Lut) and Zea). For the analysis of Fig. 5, variable contributions of the different carotenoids are anticipated according to their relative amounts generated in the corresponding samples. The cation signal of Lut can thus be expected to be approximately two times larger than that of Vio (see pigment analysis in Table 1). On the other hand, the transiently populated S_2 states of the different carotenoids interact differently with the second pulse. For example, the S_2 population of Vio may have a larger spectral overlap with the second pulse, since its $S_2 \rightarrow S_N$ band is blue-shifted compared to that of Lut. Thus, assuming that the first pulse excites approximately the same amount of carotenoids, the amount of cation generation is strongly dependent on the second pulse. As a result, the correlation between the amount of neutral carotenoids and the amount of cations generated by means of R2P2CI is lost. Nevertheless, all these phenomena have influence only on the amplitude of the cation band and not on λ_{max}. Therefore, subtraction of CP29 cations generated by R2P2CI from CP29-Zea results in a cancellation of Vio, Neo and Lut cation contributions, which contribute roughly equally in CP29 and CP29-Zea samples (Table 1). Only a positive signal remains corresponding to the increase of Zea$^{\bullet+}$ (Fig. 5).

Because of the difference in the amount of Zea bound to those samples, and the differences observed also in our TA measurements, we can attribute the transient spectral maximum at 980 nm to Zea$^{\bullet+}$, which is 20 nm blue shifted compared to the carotenoid cation which was previously identified during qE in intact thylakoids. As pointed out in previous studies [25], upon qE induction PsbS may associate with the mLHC antenna providing a highly polar environment that strongly affects the photophysical properties of Zea during qE induction. A formation of such a PsbS-mLHC complex may also explain the 20 nm blue shift of Zea$^{\bullet+}$ in mLHC compared to thylakoids.

In LHC-II the absorption band of Zea$^{\bullet+}$ [25] has been found at 983 nm. The corresponding band in CP29 Zea$^{\bullet+}$ slightly shifts to higher energies compared to LHC-II, which can be the result of different interactions of Zea$^{\bullet+}$ with the protein environment in LHC-II compared to CP29.

5. Conclusions

This paper presents two-photon two-color experiments in combination with Chl lifetime measurements on individual refolded minor light-harvesting complexes (CP24, CP26 and CP29), resembling most close native pigment content, allowing for additional critical evaluation of the role of Zea and Zea$^{\bullet+}$ in the qE component of NPQ. The pump-probe study undoubtedly showed that the presence of Zea in the investigated light-harvesting complexes has no influence on the Chl excited state lifetime. Moreover, the two-photon two-color study clearly shows the spectral position of Zea$^{\bullet+}$ in isolated CP29 at 980 nm, i.e. 20 nm blue-shifted compared to the carotenoid cation which was previously identified during qE [9], and 3 nm blue shifted compared to LHC-II Zea$^{\bullet+}$ [25].

Acknowledgements

This work has been supported by the SFB 472 ("Molecular Bioenergetics"), as well as by the "Center for Membrane Proteomics" of the University of Frankfurt. A.D. is supported by the Deutsche Forschungsgemeinschaft as an "Emmy Noether" fellow, and T.B. by the fellowship SFRH/BD/21440/2005 from Fundação para a Ciência e Tecnologia.

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