Chlorophyll fluorescence quenching by xanthophylls

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The interactions of the xanthophylls zeaxanthin, antheraxanthin and violaxanthin with chlorophyll, which are relevant for the chlorophyll fluorescence quenching in the non-photochemical quenching (NPQ) process in green plants, are investigated by means of quantum chemical methods. In particular, we use a hybrid approach consisting of time-dependent density-functional theory (TDDFT) and configuration interactions singles (CIS), since present-day TDDFT alone fails in describing relevant long-range charge-transfer states. Calculation of the energetically lowest excited states of a zeax–chl dimer along the intermolecular distance coordinate shows that two mechanisms for chlorophyll fluorescence quenching are possible: quenching via excitation-energy transfer and electron-transfer quenching. The relevance of both mechanisms is discussed by comparison to corresponding anthera–chl and vio–chl dimers and the dependence of the mechanism on the geometrical arrangement of the dimers is illuminated. It is pointed out that the typical absorption band of the zeaxanthin radical cation can be used to experimentally determine whether a zeax–chl dimer is present during NPQ.

I. Introduction

Chlorophylls and carotenoids are the elementary building blocks of the photosynthetic apparatus of plants and purple bacteria. The role of the antenna chlorophylls is to absorb sunlight and to funnel the excitation energy to the reaction center and, thereby, to trigger the photosynthetic reactions. Carotenoids generally possess one energetically low-lying dark excited state (S1) and a slightly higher lying bright excited state (S2), which let carotenoids fulfill several functions in photosynthesis. Three major ones have been established:

(1) Antenna function. Carotenoids strongly absorb light in the 400–500 nm region, where the light absorption of chlorophylls is weak. Consequently, the overall efficiency of light harvesting of the photosynthetic apparatus is significantly increased through the presence of carotenoids in the antenna systems. The carotenoids are excited into their bright S2 state, and the electronic excitation energy is efficiently transferred from the carotenoids to the chlorophylls via singlet-singlet excitation energy transfer processes.

(2) Photoprotection. Triplet states of the chlorophylls and the generation of singlet oxygen are inevitable byproducts of photosynthesis. These highly reactive species can cause severe damage to the organism and lead, in the worst case, to its death. Carotenoids, protect the organism against this damage, since they quench chlorophyll triplet states and singlet oxygen very efficiently due to a low-lying excited triplet state, which is typical for carotenoids. The carotenoid triplet state decays non-radiatively into the singlet ground state via spin conversion under generation of heat.

(3) Energy dissipation. Under high-light conditions when the photon flux to the reaction center exceeds its turn-over capacity, the generation of chlorophyll triplet states and the resulting production of singlet oxygen can increase dramatically. The organism protects itself against this scenario through a mechanism usually referred to as non-photocchemical quenching (NPQ), in which carotenoids have been shown to be involved. In contrast to the photo-protection function of the carotenoids, where dangerous byproducts are quenched, excess singlet excitation energy is directly dissipated by NPQ to prevent the increased generation of the byproducts under high-light conditions.

Upon high-light illumination over several minutes NPQ is switched on and excess singlet excitation energy is efficiently dissipated. Although NPQ has been phenomenologically documented for years, a detailed understanding of the molecular mechanism of this fundamental photosynthetic process remains elusive. Today, it is well established that a trans-membrane pH-gradient in the thylakoid is a prerequisite for NPQ, which triggers the conversion of the carotenoid violaxanthin (vio) to antheraxanthin (anthera) and zeaxanthin (zea) via the so-called xanthophyll cycle. Recent experiments have shown, that NPQ can not be activated when the xanthophyll cycle is blocked and only vio is present in PSII. Organisms which contain only anthera show only weak NPQ. Furthermore, a special pigment-binding protein (PsbS) has been found to be essential for NPQ. NPQ is in general a very complex and complicated process, in which many factors play important roles and it remains one of the last fundamental photosynthetic processes for which no molecular mechanism has yet been established.

A generally discussed and appealing molecular mechanism for the dissipation of the excess excitation energy in NPQ is singlet excitation energy transfer from the excited Qy state of the chlorophyll to the S1 state of zeaxanthin, which is known to decay non-radiatively with a lifetime of about 10 ps. It is believed that the conversion of violaxanthin to zeaxanthin leads to a decrease of the energy of the S1 state, since vio contains only nine conjugated double bonds while zeax has eleven. The fact that NPQ only works with zeax and not with vio requires the S1 state of vio to be energetically higher than the Qy state of the chlorophyll, while the S1 state of zeax should be lower. This very appealing mechanism is corroborated by recent experimental findings, which suggest that the S1 state of zeaxanthin is directly involved in NPQ. However, the experimental evidence for
the relative energetic location of the S₁ states of the xanthophylls is equivocal. Although some experiments support the proposed mechanism, a different investigation indicates that vio, anthera and zeaxanthin possess S₁ state energies lower than Qₓ, which is in contradiction to the proposed mechanism.

A second possible molecular mechanism for NPQ which is discussed in the literature is the formation of a quenching complex during the induction time of the process, which is proposed to be a zeaxanthin–chlorophyll dimer. However, no experimental evidence for the existence of a zeaxanthin–chlorophyll dimer during NPQ has yet been obtained. One can expect that formation of a dimer increases the efficiency of energy transfer processes and has substantial influence on the excited states of zeaxanthin–chlorophyll which may make new non-radiative decay channels for the Qₓ states of the chlorophylls accessible. Indeed, another possible mechanism for chlorophyll fluorescence quenching has recently been observed, when linked carotenoporphyrins were experimentally investigated. Linked carotenoporphyrins can be seen as model systems for carotenoid–chlorophyll dimers as they might be present in the NPQ process. In these experiments it has been shown that chlorophyll fluorescence quenching occurs via electron-transfer quenching, in which an electron is transferred from the carotenoid to the chlorophyll. This mechanism can equally well be described as non-radiative decay into an energetically low-lying charge-transfer (CT) state. Estimates of the ionization potentials of the involved carotenoids and the electron affinity of the linked porphyrins substantiate the proposed electron-transfer quenching mechanism. Since this process leads to the generation of a porphyrin radical anion and a carotenoid radical cation, the process can be monitored by the observation of the typical absorption of the carotenoid radical cation in the near infrared region. Recently, a similar experiment has been performed by Polivka et al. who identified a spheroidene radical cation in the light-harvesting complex of purple bacteria by transient absorption spectroscopy upon excitation of spheroidene.

In a previously published communication, we have shown that a zeaxanthin–chlorophyll CT state is lower in energy than the Qₓ state of chlorophyll and the S₁ state of zeaxanthin in a cofacial zeaxanthin–chlorophyll dimer at intermolecular distances closer than 5.5 Å. Consequently, excitation-energy transfer as well as electron-transfer quenching is in principle possible during NPQ if a zeaxanthin–chlorophyll dimer is formed. Here, we outline the applied theoretical methodology in detail and point out the failure of TDDFT in describing CT states (Section II). Furthermore, we discuss the relevance of both alternative quenching mechanisms by comparison of the zeaxanthin–chlorophyll dimer with analogous antheraxanthin–chlorophyll and violaxanthin–chlorophyll dimers (Section III B) and study the dependence of the quenching mechanism on the dimer geometry in detail (Section III C). Since the identified CT states are unique to the dimers, we suggest to experimentally search for these states during NPQ to prove the existence of a zeaxanthin–chlorophyll dimer in this process (Section III D). We conclude the paper with a summary of our major results (section IV).

II. Theoretical methods

Our theoretical investigation comprises the calculation of the electronic properties of the individual xanthophylls and chlorophyll, as well as the composition of possible xanthophyll–chlorophyll dimer structures and calculation of their electronic excited states as a function of the intermolecular distance. All calculations have been performed in the framework of the Q-Chem package of programs. Initially the geometries of zeaxanthin, antheraxanthin and violoxanthin (Fig. 1) have been optimized at the theoretical level of standard ground state density-functional theory (DFT) employing the Becke3-Lee-Yang-Parr (B3LYP) functional, which is well known to yield reliable ground state geometries. Within the geometry

Fig. 1 Structures of the xanthophylls violoxanthin, antheroxanthin and zeaxanthin, and the Chlorophyll a model complex used in the calculations.
optimization step the 6-31G* basis set was used. Within our calculations Chlorophyll a is modeled by a smaller complex (chl) which consists of the chlorin ring without the side chains (Fig. 1). This is a reasonable approximation since the side chains have only negligible effects on the lowest excited states that we are interested in, which are localized on the chlorin ring. The geometry of the model chl complex has also been optimized independently at the level of DFT using the B3LYP functional and the 6-31G* basis set. The obtained geometrical parameters agree well with crystal structure data and previously calculated parameters.

A. Choice of exchange–correlation functional for excited state calculations

For the calculation of the excited states of the xanthophyll–chlorophyll dimers we employ time-dependent-DFT (TDDFT), which is at present the most accurate and applicable method for excited states of such large molecules. In this investigation we use a variant of TDDFT, namely its Tamm–Dancoff approximation (TDA), since it has been pointed out previously that TDA/TDDFT yields very good results for linear polynomes. Particularly, full TDDFT with common functionals gives the wrong energetic order of the S_1 and S_2 states of carotenoids, while application of TDA/TDDFT (simply TDA from now on) yields the correct one: S_1 lower than S_2. A crucial step in DFT and TDDFT studies is the choice of the excitation energies, and the third state (3 A) is not found at all. In the CIS calculation the next excited singlet state is Q_x with TDA/BLYP, a lower bound to the excitation energy of the lowest CT state, 

\[ E_{\text{CT}} = \text{IP}_{\text{car}} + \text{EA}_{\text{chl}} - 1/r \]

where IP_{car} is the ionization potential of the carotenoid, EA_{chl} is the electron affinity of chl and 1/r is the electrostatic attraction between them. In this equation the zea radical cation and chl radical anion are treated as point charges, which of course may become relevant for chlorophyll fluorescence quenching. Before the quantum chemical calculation of the excited states with TDA/BLYP, a lower bound to the excitation energy of the lowest CT state, \( E_{\text{CT}} \), in which an electron is transferred from the carotenoid to chlorophyll can simply be estimated via

<table>
<thead>
<tr>
<th>State</th>
<th>Energy/eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll</td>
<td></td>
</tr>
<tr>
<td>1 A (Q_y)</td>
<td>2.552</td>
</tr>
<tr>
<td>2 A (Q_y)</td>
<td>3.376</td>
</tr>
<tr>
<td>3 A</td>
<td>—</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td></td>
</tr>
<tr>
<td>S_1</td>
<td>4.518</td>
</tr>
<tr>
<td>S_2</td>
<td>2.432</td>
</tr>
</tbody>
</table>

^a^ Taken from refs. 40 and 41. ^b^ Taken from refs. 18,20,21.

B. Calculation of charge-transfer states

Although TDA/BLYP performs very well for the calculation of the valence-excited states of the dimers, i.e. states which are localized on one molecule of the dimer and which are energetically well below their ionization potentials, TDDFT employing standard local and gradient-correct xc-functionals has severe problems in describing charge-transfer (CT) states. In the investigated dimer, when the carotenoid and zeaxanthin approach each other, CT states drop in energy due to the electrostatic interaction between the charged molecules and may become relevant for chlorophyll fluorescence quenching. In analogy to CIS, TDA/B3LYP does not find the third energetically low lying excited state of chl. The similar behavior of CIS and TDA/B3LYP can be attributed to the HF exchange part in the hybrid B3LYP xc-functional. In contrast, the gradient corrected functional BLYP and the local functional SVWN perform very well. The three low lying states of chl as well as the states of zeaxanthin, in particular the relevant S_1 state, are obtained within an error of about 0.2–0.3 eV (Table 1). It is important to note that the states which are relevant for the investigation of chlorophyll fluorescence quenching are all shifted towards higher energies than the experimentally determined values, i.e. the relative energies can be expected to be very reliable, probably with an error of less than 0.2 eV. According to our study, BLYP and SVWN yield results of essentially same quality in this particular case. Since we encountered convergence problems with the SVWN functional in many dimer calculations, we have chosen the BLYP functional to be the standard xc-functional in the excited state calculations of the xanthophyll–chlorophyll dimers. Here we should point out that the conclusions reached regarding the quality of the different functionals are valid only for the particular systems considered here and that for DFT or TDDFT studies of other systems functional performance must also be carefully investigated. For instance, B3LYP has been found to perform best in a recent similar study for the calculation of excited states of CO ligated iron porphyrin.
separation coordinate, which is due to the electrostatic attraction between the cation and anion in the CT states. This failure of TDDFT and TDA to describe such long-range CT states may be attributed to the incorrect asymptotic behavior of the used BLYP functional which is closely connected to the well-known self-interaction problem.44 It has been pointed out recently that TDDFT similarly fails for spatially extended \( \pi \) systems due to important long-range effects which are not correctly accounted for by the approximate exchange-correlation functionals.45,46 However, preliminary test calculations employing TDDFT and TDA with the asymptotic correction \( \text{LB94} \)47 for the zea–chl dimer as well as for porphin dimer systems, in which similar long-range CT states occur, show only little improvement of the excitation energies of the CT states.48

Another possible explanation for the failure of TDDFT in describing long-range CT states might be a missing linear term of the “response” part of the exchange-correlation potential employing local and gradient-corrected density functionals.49

One possibility to circumvent the self-interaction problem is to use CIS for the calculation of the dimer states. However, in the previous subsection II A it was shown that CIS overestimates the excitation energies dramatically. Moreover, CIS gives the wrong energetic order for the \( S_1 \) and \( S_2 \) states of zeaxanthin, a failure that disqualifies CIS as standard method for the desired investigation. On the other hand, CIS is self-interaction free and, therefore, the potential energy curves of the CT states exhibit the correct \( 1/r \) asymptote (Fig. 2 A). Because TDDFT yields reasonable energies for the relevant lowest valence-excited states, \( Q_1 \), \( Q_2 \), \( S_1 \) and \( S_2 \), and because CIS gives self-interaction free potential energy curves, we propose to use a hybrid of both approaches to obtain a complete self-interaction free picture of the relevant excited states.

As first step, a ground state DFT/BLYP/3-21G calculation is performed for the energetically lowest CT state at a zea–chl intermolecular distance of 9 \( \text{Å} \) by exchanging the HOMO (which is located on zea) with the LUMO (which is located on chl) (see also Fig. 5) in the beta part of the wavefunction. To converge the DFT calculation onto the energetically lowest CT state we use the geometric direct minimization (GDM) technique.50 At shorter distances than 9 \( \text{Å} \) also GDM converges to the closed-shell ground state of the dimer, otherwise we could map out a potential curve this way. A self-interaction free excitation energy for the energetically lowest zea-to-chl CT state is easily obtained by subtraction of the total energies of the ground state and CT state. Following this procedure, we obtain a value of 2.58 eV for the excitation energy of the energetically lowest CT state at 9 \( \text{Å} \), which is in agreement with the previously calculated lower bound. It is worthwhile to note that the quality of the total energy of the CT state is exactly the same as the one of the ground state, since the CT state corresponds to a pure HOMO-LUMO transition with an amplitude of almost exactly 1.0 over the considered distance range, \( i.e. \) the excited state is well described by a single Slater determinant. The self-interaction free excitation energy of the CT state obtained at the level of DFT/BLYP is then used as an offset for the self-interaction free potential energy curve calculated at the CIS level. Plotting the shifted CIS curve together with the curves of the valence-excited states calculated with TDA/BLYP yields a self-interaction free picture of all relevant excited states of the dimer. The resulting curves for the zea–chl dimer are shown in Fig. 2 C. In general, we expect the error for the lowest CT state to be of the order of 0.2–0.3 eV, \( i.e. \) the same as for the valence-excited states.

### III. Chlorophyll fluorescence quenching by xanthophylls

In this section, we present the results of our theoretical investigation of the interaction between the xanthophylls zeaxanthin, antheraxanthin and violaxanthin with chlorophyll in detail. In particular, we focus on the influence of formation of xanthophyll–chlorophyll dimers, since these might be relevant for the explanation of the molecular mechanism of the non-photochemical quenching process in green plants. Before we turn to the formation of dimers, we will outline some properties of the lowest excited states of the individual xanthophylls and chlorophyll, which will help us to understand the properties of the dimers later on (Section III A).
Thereafter, possible mechanisms for chlorophyll fluorescence quenching will be discussed with respect to the intermolecular distance between the xanthophylls and chlorophyll (Section III B). The influence of the geometrical arrangement of the dimers on the quenching mechanism will be examined (Section III C). Finally, some spectroscopic properties of the CT states will be outlined and suggestions for further experiments will be made (Section III D).

A. Individual properties of chlorophyll and the xanthophylls

Ample experimental and theoretical data are available for the individual Chlorophyll \( a \) molecule, and we used these data previously (Section II A) to gauge our theoretical approach. Using the TDA/BLYP approximation and the model complex (Fig. 1), the three lowest excited states, which are the relevant states in our investigation, have values of 2.089, 2.227 and 2.254 eV (Table 1), which is in very good agreement with the experimental values and the values obtained previously by Sundholm using TDDFT in combination with the Becke–Perdew xc-functional. For the B bands, values of 3.003 and 3.325 eV are obtained. The assignment of the calculated B bands is very difficult and not unambiguously possible due to strong mixing of the states in this energy regime. Sundholm pointed out that the usual Gouterman four-orbital model used to describe the Q and B bands in porphyrins is not sufficient for Chlorophyll \( a \), and our calculations support these findings, since we also find additional states between the Q and B bands. However, inspection of the molecular orbitals involved in these “new” states reveals that they have intramolecular charge-transfer character, and as we have seen in Section II B, excitation energies of CT states are strongly underestimated in TDDFT and TDA. This might also be the case for these states appearing between Q and B band, since they are found at substantially higher energies than Q and B band when CIS is employed. This casts some doubts as to whether the Q and B bands actually exist. However, inspection of the potential energy curves along this coordinate indicates that it is a pure HOMO (highest occupied molecular orbital) transition (Fig. 5). While the HOMO is strictly located on zeaxanthin, the LUMO is spatially restricted to the chlorophyll. At the same time the S\( 1 \) state has a similar molecular structure to violaxanthin, but violaxanthin does not have the epoxy groups. Consequently, comparison of the excitation energies and IPs of the xanthophylls with those of thzea allows us to explore the influence of the oxygen and the number of double bonds on the properties of the carotenoids.

At the TDA/BLYP level, the S\( 1 \) excitation energy drops from 2.242 to 2.100 to 2.036 eV from vio (9 double bonds) to anthera (10 double bonds) and zea (11 double bonds), respectively (Table 2). The excitation energy of the S\( 2 \) state of thzea has a value of 2.268 eV, which lets us conclude that the epoxy oxygens of violaxanthin have essentially no effect on the S\( 1 \) state energy and that it is solely determined by the number of conjugated double bonds. The same effect is found for the excitation energies of the S\( 2 \) states (Table 2). Turning to the IPs, they drop from vio to anthera and to zea from 5.258 to 5.094 and 4.956 eV. The IP of thzea has a value of 5.046 eV at the level of TDA/BLYP, which lies between anthera and zea. Comparing the IPs of vio and thzea indicates that the neglect of the epoxy oxygen decreases the IP of vio by 0.2 eV owing to the electronegative nature of oxygen. The increase of the number of conjugated double bonds from 9 in thzea to 11 in zea decreases the IP by another 0.1 eV from 5.046 to 4.956 eV. In summary, the IP is lowered by 0.3 eV from vio to zea consisting of 0.2 eV from the elimination of the epoxy oxygens and by 0.1 eV through the increase of the number of conjugated double bonds.

### B. Possible mechanisms of chlorophyll fluorescence quenching

When NPQ is active, excess excitation energy present in the Q\( y \) states of the antenna chlorophylls is efficiently quenched, i.e. the excitation energy is dissipated and converted into heat. No molecular mechanism of this fundamental process has yet been established, and even the electronic excited states that are involved in this process are not unambiguously identified. To shed some light on the possible mechanisms of chlorophyll fluorescence quenching during NPQ, we investigated the interaction between zeaxanthin and chlorophyll by calculation of the electronically excited states along the intermolecular distance coordinate. For comparison, we have also studied anthera–chl and vio–chl dimers to elucidate the relevance of different quenching mechanisms. First, we focus on the results for the cofacial middle arrangement (bottom of Fig. 6) since this turned out to be the energetically most favorable arrangement in the gas phase. Later in Section III C, the dependence of the quenching mechanism on the geometry will be investigated in detail.

Let us first turn to the zea–chl dimer, since zeaxanthin has been pointed out to be essential for an efficient NPQ process. The calculated potential energy curves along the intermolecular separation coordinate for the cofacial-middle arrangement of the zea–chl dimer are displayed in Fig. 4.A. The intermolecular separation coordinate is defined as the average distance between the planes spanned by the \( \pi \)-systems of zeaxanthin and chlorophyll. The potential energy curves along this coordinate indicate that two chlorophyll Q\( y \) quenching mechanisms are energetically accessible. Since the S\( 1 \) state of zeaxanthin is lower in energy than the Q\( y \) state of chlorophyll at the level of TDA/BLYP independent of the intermolecular distance, excitation energy transfer is in principle always possible and dimer formation is not a prerequisite for this mechanism. As soon as an intermolecular separation of less than 5.5 \( \AA \) (this is the point where the CT curve (red) crosses Q\( y \) (long-dashed curve in Fig. 4.A)) is reached, the energetically lowest CT state drops below the Q\( y \) state of chlorophyll. Consequently, a second quenching pathway becomes possible, which is electron-transfer quenching. Analysis of the molecular orbitals involved in this excited CT state shows that it is a pure HOMO (highest occupied molecular orbital) to LUMO (lowest unoccupied molecular orbital) transition (Fig. 5). While the HOMO is strictly located on zeaxanthin, the LUMO is spatially restricted to the chlorophyll molecule. Therefore, non-radiative decay of the Q\( y \) state of chlorophyll into this CT state corresponds to an electron transfer from zeaxanthin to chlorophyll.

For comparison, we have also calculated the potential energy curves for the anthera–chl dimer. Experimentally it is well established that NPQ does occur if only anthera is present.
in PSII but with lower efficiency compared to the presence of zeaxanthin. As can be seen in Fig. 4B, the Q_y state of chlorophyll and the S_1 state of anthera have essentially the same energy at the theoretical level of TDA/BLYP. According to this result, excitation energy transfer from Q_y to S_1 might be possible. It is worthwhile to note that upon dimer formation the excitation energy of S_1 drops below Q_y. However, a more detailed analysis of the small energy differences in this case is not merited because they are smaller than the accuracy of the applied theoretical approach. In analogy to the zeaxanthin–chlorophyll dimer, the lowest anthera-to-chlorophyll CT state drops below the Q_y state of the chlorophyll upon formation of a anthera–chlorophyll dimer at an intermolecular distance of 5.1 Å, i.e. electron-transfer quenching is in principle possible at shorter distances compared to the zeaxanthin–chlorophyll dimer, since the IP of anthera is higher than the one of zeaxanthin (see Table 2).

Since NPQ is known not to occur when only violaxanthin is present in PSII of green plants, we have calculated also the excited states of the violaxanthin–chlorophyll dimer along the intermolecular separation coordinate in a cofacial-middle arrangement (Fig. 4C) to elucidate possible differences between the violaxanthin–chlorophyll and the zeaxanthin–chlorophyll dimers. It turns out that the excitation energy of the S_1 state (dashed curve) is significantly higher than the Q_y state (long-dashed curve) of chlorophyll, which could be expected having the results for the individual xanthophylls in mind (Section III A, Table 2). Consequently, chlorophyll fluorescence quenching via singlet-singlet excitation energy transfer into the S_1 state is energetically not possible at all intermolecular distances. By contrast, when a dimer is formed with an intermolecular separation of less than 4.8 Å, non-radiative decay into the lowest violaxanthin–chlorophyll CT state (red curve), i.e. electron-transfer quenching, becomes accessible. The fact that the CT state of the violaxanthin–chlorophyll dimer is relatively higher than the corresponding one in the zeaxanthin–chlorophyll and anthera–chlorophyll dimers (i.e. the fact that the curve crossing of Q_y and the CT state occurs at shorter distances) can be related to the increased IP of violaxanthin compared with zeaxanthin and anthera.

Summarizing our results, two different mechanisms of chlorophyll fluorescence quenching via xanthophylls must be considered: excitation energy transfer from Q_y of chlorophyll to S_1 of...
the corresponding xanthophyll and electron-transfer quenching, where an electron is transferred from the xanthophyll to chlorophyll. The efficiency and accessibility of excitation energy transfer quenching depends on the energy of the S₁ state of the xanthophyll relative to Q₁ of chlorophyll, while electron-transfer quenching depends on the IP of the xanthophyll. While excitation energy transfer quenching is possible in the considered zea–chl dimer (S₁ is lower than Q₁) and probably accessible in the anthera–chl dimer (S₁ and Q₁ have essentially the same energy), in the vio–chl dimer quenching via excitation energy transfer is not possible (S₁ is higher than Q₁). Dimer formation is not necessary for excitation energy transfer quenching to occur since the distance between the xanthophyll and the chlorophyll has only little influence on the relative energy of S₁ and Q₁ (Fig. 3 B). However, the rate of excitation energy transfer depends on the intermolecular separation, and therefore, dimer formation would enhance the efficiency of this process.

In agreement with previous findings for linked carotenoporphyrins (see the Introduction), a second quenching mechanism via electron-transfer from xanthophyll to chlorophyll is energetically accessible when a dimer is formed. The minimum intermolecular separation at which electron-transfer quenching becomes possible depends on the IP of the xanthophylls and decreases from 5.5 Å in the zeaxanthin–chl dimer to 5.1 Å in the anthera–chl dimer and 4.8 Å in the vio–chl dimer. In contradiction to the experimentally observed quenching behavior of the different xanthophylls, this electron-transfer quenching is in principle accessible for all xanthophyll–chlorophyll dimers at the given minimal distances. One can now speculate that intermolecular separations as short as 4.8 Å can not be reached within the sterically restricted environment of the protein and that, therefore, only zeaxanthin–chl and anthera–chl show chlorophyll fluorescence quenching and vio–chl does not. Equally well it is possible that electron-transfer quenching is very inefficient and excitation energy quenching is necessary for NPQ. We cannot, at this stage, draw a definite conclusion as to whether excitation energy transfer or electron transfer is the primary mechanism for chlorophyll fluorescence quenching in NPQ. However, we can conclude that, if a zeaxanthin–chl dimer is formed during the induction time of the NPQ process, both quenching mechanisms are energetically accessible and will participate in the quenching process.

C. Influence of geometrical arrangement on the quenching mechanism

The investigation of possible quenching mechanisms has shown that for a zeaxanthin–chl dimer in a particular cofacial-middle geometry two different quenching pathways are possible. However, no experimental proof has yet been given for its existence, and thus, no experimental data for the geometrical structure of such a dimer is available. One important step of our theoretical investigation of xanthophyll–chlorophyll interactions is therefore to find reasonable geometrical arrangements between the xanthophylls and the chlorophyll, and the investigation of the influence of the specific structure on the quenching mechanisms outlined in the previous Section III B. In this context, it is not useful to rely on experimental crystal structures since we assume that during the induction time of NPQ a substantial geometrical rearrangement takes place within the proteins.

In addition to the previously described cofacial-middle arrangement, three other geometrical arrangements have been examined in detail which can be seen as limiting cases for possible dimer structures (Fig. 6). (1) a vertical-middle arrangement, where the π-systems of zeaxanthin and chlorophyll are perpendicular to each other, and the chlorophyll is placed above the center-of-mass of zeaxanthin. In this arrangement the intermolecular separation coordinate is defined as the average distance between the plane of the π-system of zeaxanthin and the closest carbon atoms of the chlorophyll model complex. (2) a cofacial-end arrangement, in which the π-systems are parallel and the chlorophyll is centered above one terminal ring of zeaxanthin. (3) a cofacial arrangement in which the chlorophyll is moved parallel to the π-system of zeaxanthin by 4 Å from its position in the cofacial-end arrangement towards its position in the cofacial-middle arrangement. In this geometry as well as in the cofacial-end arrangement, the intermolecular separation coordinate is defined as the average distance between the planes spanned by the π-systems of zeaxanthin and chlorophyll.

Calculation of the excited states of the different dimer structures with respect to the intermolecular distance coordinate employing the proposed hybrid approach yields the results summarized in Fig. 7. In the vertical-middle arrangement (Fig. 6), quenching via excitation-energy transfer is possible since the energy of the S₁ state of zeaxanthin is lower than the Q₁ state of chlorophyll. In contrast, electron-transfer quenching is not possible in this arrangement since the energetically lowest CT state (red curve in Fig. 7) is well above the Q₁ state of the chlorophyll. This is due to the distance between the HOMO of zeaxanthin and the LUMO of chlorophyll, which are the electron donating and accepting molecular orbitals in the transfer process, respectively. Compared to the cofacial-middle arrangement, the effective distance between these orbitals is larger in the vertical-middle arrangement resulting in a decreased electrostatic attraction within the CT state and a larger excitation energy. The effective distance explains nicely why electron transfer is possible in the cofacial-middle arrangement but not in the vertical-middle arrangement. Turning to the cofacial-end arrangement (Fig. 6), excitation energy transfer is possible and electron transfer is, in analogy to the vertical-middle arrangement, energetically not accessible. In this arrangement, the chlorophyll is located above one of the terminal six-membered rings of zeaxanthin, where the HOMO, the orbital where the transferred electron comes from, has no contributions.

**Fig. 6** Different geometrical arrangements for the xanthophyll–chlorophyll dimers. We studied a vertical middle arrangement (top), a cofacial end arrangement (middle) and a cofacial middle arrangement (bottom).
becomes possible. To answer this question we constructed the two limiting cases is the first for which electron transfer is possible quenching mechanism.

Since electron transfer is possible in the cofacial-middle arrangement but not in the cofacial-end arrangement, a natural question to ask is which cofacial arrangement between these two limiting cases is the first for which electron transfer becomes possible. To answer this question we constructed the cofacial arrangements by starting from the cofacial-end geometry and moving the chlorophyll model complex relative to zeaxanthin towards its position in the cofacial-middle arrangement. The first dimer which can quench chlorophyll fluorescence via excitation energy transfer and electron-transfer quenching is one in which the chlorophyll is moved 4 Å from the cofacial-end position. The corresponding potential energy curves along the intermolecular separation coordinate are given in Fig. 7. For all other cofacial arrangements between this and the cofacial-middle one both quenching mechanisms are energetically accessible.

Summarizing our findings, chlorophyll fluorescence quenching via excitation energy transfer is energetically accessible for all geometrical arrangements and in principle at all distances. Electron-transfer quenching can occur only in cofacial dimers in which the π-systems of zeaxanthin and chlorophyll sufficiently overlap.

**D. Spectroscopic manifestation of the charge-transfer states**

As stated previously, no experimental proof for the existence of a zeaxanthin–chlorophyll complex during NPQ has yet been given. In the previous section, we have shown that electron transfer from zeaxanthin to chlorophyll is possible quenching mechanism when a cofacial dimer is present during NPQ, since a corresponding zeaxanthin–chlorophyll CT state is only then energetically accessible. We suggest that this charge-transfer state may be an experimental signature of dimer formation during NPQ, since the CT state is unique to the dimer. If such a dimer is present during NPQ, the initially excited Q-state will decay at least partially into the CT state, i.e. an electron will be transferred from zeaxanthin to chlorophyll. This electron transfer leads to the formation of a zeaxanthin radical cation and a chlorophyll radical anion.

Carotenoid radical cations are known to absorb strongly in the near infrared region of the spectrum and according to a TDA/BLYP calculation, the zeaxanthin radical cation possesses two electronic transitions at wavelengths of about 750 and 1290 nm with large oscillator strengths. Consequently, a desirable future experiment would be a pump-probe experiment in which the Q-state of the antenna chlorophyll is pumped and the zeaxanthin radical cation absorption is probed while NPQ is active. If a signal corresponding to the radical cation can be observed only during NPQ, the existence of a zeaxanthin–chlorophyll dimer during NPQ would be proven. Similar experiments have been performed recently, e.g. in which the antenna chlorophylls of PSII were excited and the S1 → S0 region probed, or in which the S2 state of spheroidene in LH2 was pumped and the transient absorption spectrum recorded. In this latter experiment a spheroidene radical cation was identified. The radical cation is formed within about 200 fs and decays within 8 ps.

**IV. Summary and conclusions**

Non-photochemical quenching protects green plants from photo-oxidative damage by efficiently dissipating excess excitation energy. The key step in this process is the efficient quenching of chlorophyll fluorescence. As yet, no molecular mechanism for NPQ has been established. To shed some light onto possible mechanisms of chlorophyll fluorescence quenching in NPQ, the interaction of the xanthophylls zeaxanthin (zea), antheraxanthin (anthera) and violaxanthin (vio) with chlorophyll (chl) has been studied theoretically. In particular, the influence of dimer formation on the excited states of the species was of primary interest. For this objective we studied the excited states of xanthophyll–chlorophyll dimers along the intermolecular distance coordinate by means of a hybrid approach combining time-dependent density-functional theory (TDDFT) and configuration interaction singles (CIS). We used this hybrid approach, since TDDFT employing standard exchange-correlation functionals fails in describing...
charge-transfer (CT) states which are relevant for the understanding of energy and electron transfer processes in the investigated systems. The excitation energies of long-range CT states are critically underestimated by 1–2 eV, and the potential energy curves along the intermolecular distance coordinate do not exhibit the physically correct 1/r asymptotic behavior. Calculation of the excited states of the xanthophyll-chlorophyll dimers along the intermolecular separation coordinate revealed that two different quenching mechanisms are possible when a zeaxanthin-chlorophyll dimer is formed. On the one hand, quenching via excitation energy transfer from Qy of chl to S0 of zeaxanthin is possible at all intermolecular distances, and consequently, dimer formation is not a prerequisite for this mechanism although it would enhance its efficiency. On the other hand, if a dimer is formed, electron-transfer quenching becomes possible as well since a zeaxanthin-to-chlorophyll CT state becomes lower than the Qy state at an intermolecular distance of 5.5 Å. Comparison with corresponding antheraxanthin-chlorophyll and violaxanthin dimers shows that the experimental findings can be explained by excitation-energy transfer alone and that dimer formation is in principle not necessary. However, if a dimer is formed during NPQ, both possible quenching mechanisms must be considered. Because of the charge separation the CT state possesses a very large static dipole moment compared to the Qy and S0 state of chlorophyll and the xanthophylls, respectively. Therefore the CT states will be energetically strongly stabilized relative to the other states if the dimer is in a polar medium such as in the protein environment. Investigation of the geometry dependence of the quenching mechanisms revealed that the accessibility of quenching via excitation-energy transfer does not depend on a specific geometry of the zeaxanthin–chlorophyll dimer and that it is in principle always possible. On the contrary, electron-transfer quenching is sensitive to the geometrical arrangement of the zeaxanthin–chlorophyll dimer and can only occur in cofacial arrangements where the π systems of zeaxanthin and chlorophyll sufficiently overlap. In our calculations, these geometries turned out to be energetically more favorable than the other investigated ones and thus it is not unlikely that they are formed during NPQ. On the contrary, the protein environment can have a significant impact on the geometrical structure of the dimer and, in particular, twisted geometries of the xanthophyll dimer might be present. This can also have an influence on the accessibility of the quenching mechanisms.

Since the CT states are energetically only accessible when a dimer is formed, i.e. only then electron-transfer quenching can occur, the CT states can help to experimentally answer the question whether a dimer is formed during NPQ or not. We suggest to use the characteristic absorptions of the zeaxanthin radical cation, which is part of the CT state, at 750 and 1290 nm as a signature of the dimer during NPQ.

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