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Initial excited-state relaxation of the bilin chromophores of phytochromes: a computational study[†]

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[†] Electronic supplementary information (ESI) available: Cartesian coordinates of optimized structures.

Graphical abstract: Quantum chemical calculations show that the intrinsic reactivity of the bilin chromophores of phytochromes is qualitatively very different from their reactivity in the protein, and even favors a different photoisomerization reaction than that known to initiate the photocycles of phytochromes.

Abstract: The geometric relaxation following light absorption of the biliverdin, phycocyanobilin and phytochromobilin tetrapyrrole chromophores of bacterial, cyanobacterial and plant phytochromes has been investigated using density functional theory methods. Considering stereoisomers relevant for both red-absorbing Pr and far-red-absorbing Pfr forms of the photoreceptor, it is found that the initial excited-state evolution is dominated by torsional motion at the C10–C11 bond. This holds true for all three chromophores and irrespective of which configuration the chromophores adopt. This finding suggests that the photochromic cycling of phytochromes between their Pr and Pfr forms, which is known to be governed by *Z/E* photoisomerizations at the C15–C16 bond, relies on interactions between the chromophore and the protein to prevent photoisomerizations at C10–C11. Further, it is found that the uneven distribution of positive charge between the pyrrole rings is a major factor for the photochemical reactivity of the C10–C11 bond.

Keywords: Photoreceptors, Linear tetrapyrroles, Adiabatic excited-state geometries, Isomerization reactions, Photocatalysis, Time-dependent density functional theory

Introduction

Phytochromes are a family of biliprotein photoreceptors first discovered in plants but present also in cyanobacteria, fungi and nonphotosynthetic bacteria.¹ Responsive to red and far-red light through the absorption of their linear tetrapyrrole (bilin) chromophores (Figure 1), these photoreceptors exist in two photochromic forms known as Pr and Pfr.² By switching between these forms, phytochromes regulate a variety of physiological responses, ranging from seed germination in plants to phototaxis in bacteria.^{3,4} In most phytochromes the red-absorbing Pr form ($\lambda_{\text{max}} \sim 660$ nm) is predominant in the dark-adapted ground state and the far-red-absorbing Pfr form ($\lambda_{\text{max}} \sim 730$ nm) is predominant in the biologically active state.

Despite long-standing efforts it has proven difficult to establish the molecular mechanism by which Pr is converted into Pfr.^{3,4} This is largely a consequence of the scarcity of structural data to provide insight into the interplay between the chromophore and the protein as the reaction progresses. In fact, while a number of X-ray crystal and NMR solution structures of the chromophore-binding domains of Pr phytochromes are available,^{5–9} with important implications for understanding the initial stages of the reaction, such structures of the Pfr form have become available only recently.^{10,11}

The Pr→Pfr conversion proceeds via a number of metastable intermediates with distinct spectral properties.^{12–14} As for the primary photochemical event, which produces the first intermediate (Lumi-R) within a few tens of ps after light absorption,^{15,16} it is widely recognized that this step is achieved by a *Z*→*E* photoisomerization of the bilin chromophore, occurring at the C15–C16 bond of the methine bridge between rings C and D.^{3,4} Such a mechanism is supported by, *e.g.*, NMR data on phytochrome chromopeptide fragments,^{17,18} resonance Raman (RR) spectroscopy studies,^{19–21} UV-Vis spectra of phytochromes assembled with sterically locked bilins,²² magic-angle spinning NMR studies,²³ and recent time-resolved RR experiments monitoring the formation of Lumi-R at sub-picosecond resolution.²⁴ Furthermore, the aforementioned Pr crystal structures of bacterial^{5–7} and cyanobacterial⁸ phytochromes indicate that *Z*→*E* photoisomerization at the CD bridge is favored over the corresponding reactions at the AB and BC bridges because of the tight packing of rings A–C by the protein. Ring D, in contrast, resides in a pocket providing ample space for rotation around the C15–C16 bond. Another factor that may render the CD bridge more reactive than the AB and BC bridges is the anchoring of the chromophore to the apoprotein, which occurs through a thioether linkage to ring A and which would seem to impose mechanical resistance towards rotation that gradually decreases from ring A to ring D.

In a recent study, density functional theory (DFT) calculations were performed to establish the intrinsic reactivity of phytochromobilin (PΦB), the chromophore used by plant phytochromes, towards $Z \rightarrow E$ photoisomerization at all three methine bridges in the parent Pr state.²⁵ Focusing on the C5-*Z*,*anti* C10-*Z*,*syn* C15-*Z*,*anti* (*ZaZsZa*) stereoisomer predicted by RR data^{26,27} and identifying the preferred reaction channel in the absence of steric effects and specific interactions with the protein, it was found that such conditions allow isomerization at the C10–C11 bond to substantially dominate over the (biologically preferred) isomerization at the C15–C16 bond.²⁵ This finding suggests that the protein plays a decisive role not only in promoting a very quick photoreaction at C15–C16 (within 3 ps according to a recent estimate²⁴), but also in preventing the reaction from rather taking place at C10–C11. Importantly, however, the physical origin of the photochemical reactivity of the C10–C11 bond of PΦB remains unclear.

In the present study, we report the same type of quantum chemical calculations to rationalize the previous results and establish whether this behavior of PΦB is shared by the biliverdin IXα (BV) and phycocyanobilin (PCB) chromophores of bacterial and cyanobacterial phytochromes, respectively. Moreover, by also investigating the molecular motion induced by light absorption of BV, PCB and PΦB in geometries relevant for the biologically active Pfr state, we address what role is required of the protein to accomplish the *reverse* $E \rightarrow Z$ photoisomerization that initiates the Pfr→Pr deactivation process. Finally, we propose a very simple model for how the protein may modulate the photochemical reactivity of BV, PCB and PΦB.

Methods

The calculations considered the stereoisomers of BV, PCB and PΦB listed in Table 1. The stereoisomers for the Pr forms – *ZsZsZa* BV, *ZsZsZa* PCB and *ZaZsZa* PΦB – were chosen based on crystal structures of bacterial,^{5–7} crystal and NMR structures of cyanobacterial,^{8,9,28} and RR studies of plant^{26,27} phytochromes, respectively. Note that a crystal structure of a plant phytochrome is yet to be reported and that the configuration of the AB bridge in PΦB (*Za*) is different from that in BV and PCB (*Zs*). The stereoisomers for the Pfr forms, in turn, were chosen so as to account for both the possibility that the only change in chromophore configuration during the Pr→Pfr conversion is that due to the $Z \rightarrow E$ photoisomerization at C15–C16, and the possibility^{26–31} that a complementary *thermal* single-bond isomerization occurs at C5–C6 during the transition from Lumi-R to Pfr. Based on the experimentally

observed Pr stereoisomers, these two scenarios implicate two sets of possible Pfr stereoisomers, both of which were subjected to calculations: {*ZsZsEa* BV, *ZsZsEa* PCB, *ZaZsEa* PΦB} and {*ZaZsEa* BV, *ZaZsEa* PCB, *ZsZsEa* PΦB}, respectively.

The calculations were carried out using models of BV, PCB and PΦB with the conjugated π -systems fully intact. To reduce the computational effort, however, the C3 thioether linkage, the C8 and C12 propionic carboxyl groups, and the C2, C7, C13 and C17 methyl groups were replaced by hydrogen atoms (see Figure 1 for atom numbering). These substitutions have been shown³² to have a negligible effect on the overall electronic structure of the chromophores (see also the results of complementary calculations employing larger model systems below). Based on spectroscopic evidence pertaining to both Pr and Pfr phytochromes,^{33–37} all calculations considered cationic species with all four nitrogens protonated. Using the same level of theory as a previous study,²⁵ ground (S_0) and excited-state (S_1 , the lowest excited singlet state) geometries were optimized with the B3LYP hybrid density functional in combination with the Karlsruhe SVP basis set. The accuracy of this particular level of theory was tested using a number of other density functionals and basis sets to perform complementary calculations, as further described below. The excited-state optimizations were carried out with the method of Furche and Ahlrichs,³⁸ which uses a time-dependent DFT (TD-DFT) formalism.^{39,40} The optimized ground and excited-state geometries were subjected to analytic (B3LYP/SVP S_0) and numerical (TD-B3LYP/SVP S_1) force-constant calculations, respectively, and were thereby identified as potential energy minima. All calculations were performed with the GAUSSIAN 09 and TURBOMOLE 5.7 (for optimizing excited-state geometries) program packages.^{41,42}

Throughout the paper, the NA–C4–C5–C6, C4–C5–C6–NB, NB–C9–C10–C11, C9–C10–C11–NC, NC–C14–C15–C16 and C14–C15–C16–ND dihedral angles are denoted C4–C5, C5–C6, C9–C10, C10–C11, C14–C15 and C15–C16, respectively (*i.e.*, specifying only the central bond to which the angle pertains).

Results and discussion

Assessment of quantum chemical methodology

The accuracy of the chosen level of theory (B3LYP/SVP) for investigating the photochemical reactivity of bilin chromophores was assessed by computing absorption and fluorescence maxima of the dihydrobiliverdin species, hereafter denoted DHBV, shown in Figure 2. These spectroscopic parameters

have been recorded experimentally in a chloroform solvent.⁴³ The calculations were carried out with DHBV adopting a cyclic *ZsZsZs* configuration, which is the configuration preferred by bilins in solution,³ and made use of the same side-chain substitutions as those employed for BV, PCB and PΦB, as described above. The chloroform solvent was modeled using the integral equation formulation of the polarizable continuum model (PCM),⁴⁴ with the dielectric constant set to 4.9.

Encouragingly (see Table 2), the computed 605 nm absorption maximum (vertical $S_0 \rightarrow S_1$ excitation energy) of DHBV at the B3LYP/SVP level is in very good agreement with the experimental value of 590 nm. Such agreement is rarely observed in calculations of the present type; however, it is of course possible that errors in our approach tend to cancel each other. The computed 761 nm fluorescence maximum (vertical $S_1 \rightarrow S_0$ emission energy) is also reasonably close to the experimental value, deviating by less than 0.2 eV from 690 nm. This result is particularly relevant because it indicates that B3LYP/SVP provides accurate excited-state geometries of bilin chromophores and, therefore, a reliable description of the molecular motion induced by light absorption. The nice agreement between the computed (0.42 eV) and experimental (0.30 eV) Stokes shifts reinforces this conclusion.

As for the performance of B3LYP/SVP relative to other levels of theory, the absorption and fluorescence maxima of DHBV were also calculated using B3LYP in combination with three larger basis sets, and using four other well-established (PBE0, BLYP, BP86 and τ -HCTH) and three more recently developed (M06-HF,⁴⁵ CAM-B3LYP⁴⁶ and LC- ω PBE⁴⁷) density functionals. From Table 2, we first note that the Karlsruhe (SVP and TZVP) and correlation consistent (cc-pVDZ and aug-cc-pVDZ) basis sets are of similar accuracy, and that neither increasing the basis from SVP to TZVP nor augmenting the cc-pVDZ basis with diffuse functions improves the computed transition energies. Thus, the SVP basis set appears fully adequate for the purpose of the present investigation. This finding is in line with previous studies of the PΦB chromophore.^{25,48} Turning to the choice of functional, B3LYP compares favorably with all other methods, consistently yielding results that are more accurate than BLYP, BP86 and τ -HCTH and similar to PBE0. Furthermore, none of the M06-HF, CAM-B3LYP and LC- ω PBE functionals, although having a broader range of applicability,^{45–47} provides overall better estimates of the transition energies than B3LYP.

It should be noted that the methodology is here validated based on how well it reproduces energies of the lowest excited S_1 state alone, rather than by calculating the full absorption and fluorescence spectra of DHBV and comparing with their experimental counterparts.⁴³ While calculation of the full spectra would offer further insight into the photochemistry of bilins, we believe that focusing

on the S_1 state is a more appropriate approach because previous studies have demonstrated that no other excited state is involved in the photoisomerizations under investigation,^{25,49,50} although the character of the state may change during the final stages of the reactions.⁵⁰ That TD-DFT performs well for this state, which has also been reported by other authors,^{51,52} is not surprising given that the state has single-excitation $\pi\pi^*$ character and the same charge distribution between the pyrrole rings as the ground state^{25,53} not only at chromophore geometries close to the Franck-Condon (FC) region, but also at torsionally distorted chromophore geometries.^{25,53} The inability of conventional TD-DFT methods to treat excited states with appreciable charge-transfer character^{54,55} is therefore of no significance for the present study. In fact, for a reduced model of the bilin chromophore comprising rings B and C only, the S_1 potential energy curve computed with TD-DFT is in excellent agreement with that computed using a more advanced *ab initio* method (CASSCF) for more than 50° rotation around the methine bridge.²⁵ Hence, TD-DFT appears to be a viable tool for exploring the photochemical reactivity of bilin chromophores. This is fortunate because TD-DFT is presently the only correlated quantum chemical method available for which excited-state geometry optimizations of systems as large as bilins are practical. Since TD-DFT would not perform well for the *fully* torsionally distorted ($\sim 90^\circ$) chromophore geometries at which S_1 may acquire the character of a twisted intramolecular charge-transfer state,⁵⁰ the present study is exclusively concerned with mapping the regions of the bilin excited-state potential energy surfaces where this deficiency does not come into play, as further outlined below.

Geometric relaxation from the Franck-Condon region

The molecular motion induced by light absorption of the bilin chromophore is the result of geometric relaxation from the vertically excited FC region to the nearest minimum on the excited-state potential energy surface. Accordingly, this motion can be assessed by comparing (see Table 3 and Figure 3) the optimized ground and excited-state geometries of BV, PCB and PΦB. Starting with the ground-state geometries, we first note that these are best described as hybrids of the resonance structures **II** and **III** of Figure 1, with little contribution from **I** and **IV**. This feature is also reflected by the charge distribution, with most of the net positive charge residing at rings B and C.^{48,56} In each system, the BC bridge is comprised of nearly identical C9–C10 and C10–C11 bonds, whereas the AB and CD bridges show a distinct differentiation between single (C5–C6 and C14–C15, respectively) and double (C4–C5 and C15–C16, respectively) bonds. The single-bond character of C5–C6 and C14–C15 implies that isomerizations at these sites would not require the system to be promoted to the excited state, but would

rather proceed thermally. Hence, only the C4–C5, C9–C10, C10–C11 and C15–C16 sites are conceivable for photoisomerization. However, in all three chromophores and for both Pr and Pfr stereoisomers thereof, C4–C5 and C15–C16 retain and C9–C10 gains double-bond character in the excited state, which renders photoisomerizations at these sites less probable in the absence of a surrounding protein. Specifically, FC relaxation lengthens the C4–C5 and C15–C16 bonds by 0.01–0.02 Å only and *shortens* the C9–C10 bond by up to 0.03 Å. Furthermore, the resulting torsional motion at these sites amounts to no more than 0–5° (C4–C5), 1–7° (C15–C16) and 1–5° (C9–C10), respectively.

The C10–C11 bond, on the other hand, develops single-bond character in the excited state, which facilitates photoisomerization at this site. In fact, for both Pr and Pfr forms of all three bilins, FC relaxation lengthens the C10–C11 bond by 0.04–0.05 Å and induces torsional motion of 6–15°. Accordingly, while the Pr→Pfr conversion is initiated by a *Z*→*E* photoisomerization at C15–C16 in the protein,^{3–8,17–24} and the reverse Pfr→Pr deactivation process consequently relies on an *E*→*Z* photoisomerization at the same site, *all* bilin chromophores used by phytochromes are in isolated conditions more prone to start isomerizing at C10–C11. This holds true *irrespective* of which configuration (Pr or Pfr) the chromophores adopt. These findings indicate that interactions between the chromophores and the protein are critical for the photochemistry and photochromism exhibited by phytochromes and, particularly, for the chromophores to isomerize at C15–C16 rather than C10–C11. In this context, the differences in packing of the pyrrole rings shown by the available crystal structures^{5–8} appear to be more important than previously believed, allowing for unperturbed rotation of ring D around the C15–C16 bond^{3,4} but also posing steric hindrance for isomerizations at the AB and BC bridges.

Given that the FC relaxation changes the BC bridge from being essentially symmetric (equal C9–C10 and C10–C11 bonds) in the ground state to being distinctly asymmetric (shortened C9–C10 bond and elongated C10–C11 bond) in the excited state, it should be pointed out that complementary optimizations aimed to locate symmetric excited-state minima, or excited-state minima with the opposite features (*i.e.*, elongated C9–C10 bond and shortened C10–C11 bond), clearly indicated that no such alternative minima exist. In fact, despite using suitably modified starting geometries exhibiting either of these alternative features, the complementary optimizations reproduced exactly the original excited-state minima, which therefore constitute a solid basis for the conclusions drawn above. Moreover, to ascertain that the features of the original excited-state minima are not artefactually dependent on the density functional (B3LYP) and basis set (SVP) employed, the ground and excited-state geometries of *ZaZsZa*

PΦB were also optimized with the PBE0 hybrid functional and the larger TZVP basis set (see Table 4). Encouragingly, these optimizations reinforce the conclusion that the FC relaxation in isolated bilin chromophores is dominated by stretching of and rotation around the C10–C11 bond. Indeed, the corresponding numerical values for the changes in bond lengths and dihedral angles agree to within 0.007 Å and 2.1° with those computed at the B3LYP/SVP level. Furthermore, our previous study, which focused exclusively on the Pr form of PΦB, showed that also calculations carried out with the BP86 functional support this conclusion.²⁵

Photoisomerization paths

Having focused on the FC relaxation, it is pertinent to explore whether also the regions of the excited-state potential energy surfaces that correspond to further torsional motion reveal a tendency of isolated bilin chromophores to isomerize at C10–C11 rather than C15–C16. To this end, the excited-state potential energy surfaces of *ZsZsZa* BV (a Pr isomer) and *ZsZsEa* BV (a Pfr isomer) were mapped in greater detail by performing a series of constrained TD-B3LYP/SVP geometry optimizations, as shown in Figure 4. These calculations describe up to 60° torsional motion around the photochemically relevant C4–C5, C10–C11 and C15–C16 bonds, and were at each point carried out by enforcing a single dihedral constraint and relaxing all other degrees of freedom. The reason for not extending the calculations to include also the regions around ±90° where the systems are expected⁵⁷ to decay to the ground state is twofold. First, valuable and conclusive information on the photochemical reactivity is contained already in the regions that the calculations do include. Second, due to charge-transfer and near-degeneracy effects, it is a major challenge to properly describe these decay channels using TD-DFT. In this light, it is important to point out that a detailed analysis of charge distributions and explicit comparison with CASSCF calculations have shown^{25,53} that the magnitudes of such effects remain small in the range of dihedrals here considered, and become pronounced only closer to the orthogonal geometries.

From Figure 4, we note that torsional motion at C10–C11 continuing beyond the excited-state minima at 19.7° (*ZsZsZa* BV) and 20.0° (*ZsZsEa* BV) in the same direction as the FC relaxation (*i.e.*, towards +60°) has energy barriers of about 1 kcal mol^{−1} only, whereas torsional motion in the opposite direction (*i.e.*, towards −60°) is estimated to have barriers of at least 5–6 kcal mol^{−1}. Given that the calculations involve constrained geometry optimizations rather than minimum energy path computations and furthermore do not explore the regions beyond −60° that, judging from Figure 4, may well lie even higher in energy, these estimates are likely to be lower bounds to the true energy barriers. Hence, the

directionality of the C10–C11 motion appears to be governed entirely by the FC relaxation. The photoisomerizations at the C4–C5 and C15–C16 bonds, in turn, have barriers that for both stereoisomers and both isomerization directions amount to at least 6–11 kcal mol⁻¹. Such barriers quite significantly exceed the 1 kcal mol⁻¹ required for the photoisomerization at C10–C11, and effectively rule out efficient photochemistry at C4–C5 and C15–C16 in the absence of the protein. This finding reinforces the conclusion that the bond about which photoisomerization of bilin chromophores is easiest in isolated conditions is the C10–C11 bond, and not the C15–C16 bond known to be preferred by phytochromes. Importantly, we would like to stress that this conclusion should not be taken as support for an alternative mechanism wherein the primary event of phytochromes is achieved by a photoisomerization at C10–C11 instead of C15–C16, but should be viewed as a clear indication that the protein must substantially alter the photochemical reactivity of the bilin chromophores for the primary event to take place at C15–C16.

The possible photoisomerization routes of bilins have recently also been investigated quantum chemically by Altoè et al.⁵⁰ Focusing on the *ZaZsZa* PΦB chromophore and using the uncorrelated configuration interaction singles (CIS) method for excited-state geometry optimizations and the CASPT2 method for subsequent singlepoint calculations, these authors also found that the C10–C11 bond is intrinsically more reactive than the C15–C16 bond. Interestingly, however, their rationale was different from ours and based on the idea that the S₁–S₀ energy gap at the fully distorted chromophore geometry (~90°) along the C10–C11 route is much smaller than the corresponding energy gap along the C15–C16 route. This idea was then used as a basis for proposing a kinetic model relating the faster and slower components of the excited-state decay in the protein to aborted photochemistry at C10–C11 (because of steric hindrance) and successful photochemistry at C15–C16, respectively.⁵⁰ While the present data afford no such assignment of the different decay components, the reported S₁–S₀ energy gap along the C15–C16 route in the model by Altoè et al.⁵⁰ seems too large (17.4 kcal mol⁻¹) to readily account for the experimental observation^{15,16} that the Lumi-R intermediate is formed already within a few tens of ps after light absorption.

Assessment of model system

A potential source of error in the calculations of this work is the use of truncated chromophore models in which the propionic and methyl groups are replaced by hydrogen atoms. To ascertain that the calculations nonetheless allow for a proper description of the electronic and geometric features of the

chromophores and their structural evolution during the photoisomerization events, a set of complementary calculations using larger models that retain these groups were performed. Denoting the original model as Model I, the following additional models were considered: Model II, in which the methyl groups are retained; Model III, in which the propionic groups are retained; and Model IV, in which both the methyl and propionic groups are retained.

First, these larger models were used to calculate the absorption maxima of *ZsZsZa* BV and *ZsZsEa* BV. From Table S1 of the ESI, we note that the methyl and propionic groups contribute to a red shift in the absorption, but that the shift is small for both chromophores (≤ 0.04 and ≤ 0.10 eV, respectively). Hence, these groups do not appear to strongly influence the electronic structure of bilins, *i.e.*, Model I should be a reasonable choice of computational model in this regard.

Second, to investigate whether the methyl and propionic groups play a role in the FC relaxation (possibly manifested through steric hindrance), the ground and excited-state geometries of the four different models of *ZsZsZa* BV and *ZsZsEa* BV were optimized using B3LYP for the ground state and CIS for the excited state. Although CIS allows for much cheaper mapping of excited-state potential energy surfaces than TD-DFT, a comparison between the resulting excited-state geometries and the ground-state geometries obtained with B3LYP does not give a quantitative description of the FC relaxation, as the two sets of geometries are of different quality. However, such a comparison, which is presented in Table S2 of the ESI, does reveal the extent to which the methyl and propionic groups affect the FC relaxation. Pleasingly, we note that Model I compares very well with the larger models insofar that the estimated differences in bond lengths and dihedral angles between the ground state and the excited state throughout are in close agreement with the values obtained using the larger models. Model I therefore appears sufficiently large to provide a reliable description of the FC relaxation.

Third, and finally, approximate TD-B3LYP/SVP excited-state potential energy curves for up to 60° torsional motion around the C4–C5, C10–C11 and C15–C16 bonds of *ZsZsZa* BV were computed by performing TD-B3LYP/SVP singlepoint calculations on a series of B3LYP/SVP ground-state geometries of both the smallest (I) and the largest (IV) computational models, as shown in Figure S1 of the ESI. These calculations complement the results of Table S2 by investigating the accuracy of Model I also for twisted chromophore structures distant from the FC region. As can be seen, the potential energy curves obtained using Model I are very similar to those obtained using Model IV, with average energy differences between the two sets of data points that amount to no more than 0.4, 0.6 and 0.5 kcal mol⁻¹

for the C4–C5, C10–C11 and C15–C16 pathways, respectively. Overall, then, Model I seems just as suitable as Model 4 for exploring the photochemical reactivity of bilin chromophores.

Origin of the photochemical reactivity of the C10–C11 bond

Why are then the bilin chromophores of phytochromes more likely to start photoisomerizing at the C10–C11 bond in the absence of a surrounding protein? One possible contributing factor is the steric repulsion between rings B and C that the *Zs* configuration of the BC bridge introduces. To test this hypothesis, calculations were also carried out on the *ZaEaZs* stereoisomer of PΦB, whose BC bridge adopts an *Ea* configuration. Although not supported by recent experiments, this stereoisomer has been reported in RR studies of plant phytochrome.²¹ As can be seen from Table 3 and Figure 5, however, the geometric changes of *ZaEaZs* PΦB in the excited state are similar to those of the other bilins. Furthermore, the C15–C16 bond remains unreactive in this stereoisomer, despite the fact that the CD bridge now has a *Zs* configuration. Thus, steric effects do not seem to contribute to the photochemical reactivity of the C10–C11 bond.

Another conceivable factor is electrostatic repulsion, which should be greater between rings B and C because, as inferred by NMR data⁵⁸ and quantum chemical calculations,^{48,56} these carry larger portions of the net positive charge than rings A and D. To test this hypothesis, the ground and excited-state geometries of the Pr bilins were optimized in the presence of a chloride anion, placed and held fixed at 2 Å distances from the hydrogens of the NH moieties of rings A–C. These calculations serve a double purpose by also probing the potential photochemical roles of counterions for the reactivity of the chromophores inside the protein. In the available Pr crystal structures,^{5–8} the backbone carbonyl oxygen of an aspartate residue (Asp-207 in the DrBphP bacteriophytochrome) and the δ1 nitrogen of a histidine residue (His-260) are within hydrogen bonding distance of the A, B and C-ring nitrogens (*i.e.*, their distances to the corresponding hydrogens are about 2 Å). It is thus possible, as first argued by Rockwell et al.,³ that the partial negative charges of the aspartate backbone oxygen and the histidine δ1 nitrogen stabilize the cationic chromophores in Pr. Such a scenario has been implicated in recent flash photolysis experiments.⁵⁹

Interestingly, from Table 3 and Figure 6 we note that neutralizing the positive charge of rings B and C has a substantial effect on the FC relaxation. First, the 0.04–0.05 Å lengthening of the C10–C11 bond disappears completely (PCB and PΦB), or is reduced to 0.02 Å (BV). Hence, the propensity of isolated bilin chromophores to start photoisomerizing at C10–C11 indeed appears to be a consequence

of the uneven distribution of positive charge between the pyrrole rings. Second, the C15–C16 bond becomes more reactive. This suggests that counterions are important not only for stabilizing the cationic chromophore,^{3,59} but could also play a role for the Pr→Lumi-R photoconversion by inhibiting an unwanted (at C10–C11) and promoting the desired (at C15–C16) $Z\rightarrow E$ isomerization. Another residue of interest in this regard is the tyrosine (Tyr-176 in the DrBphP bacteriophytochrome) that lines the pocket surrounding ring D in Pr.^{5–8} In fact, mutational studies have shown that this tyrosine is essential for the function of the cyanobacterial phytochrome Cph1, as replacing it with any other residue lowers the photoconversion efficiency.^{60,61} Although beyond the scope of the present study, elucidating exactly how the tyrosine facilitates the photoconversion, perhaps by gating ring D during its rotation,^{4,60} is a worthwhile objective for future computational studies in this field of research.

Conclusions

In summary, we have performed density functional theory calculations to investigate the geometric relaxation following light absorption of the BV, PCB and PΦB chromophores of bacterial, cyanobacterial and plant phytochromes, in the absence of a surrounding protein. Using a level of theory that accurately reproduces experimental absorption and fluorescence maxima of a related dihydrobiliverdin species in chloroform,⁴³ and considering stereoisomers relevant for both the inactive Pr and the active Pfr form of the photoreceptor, it is found that the initial excited-state evolution of *all* three chromophores is in *all* stereoisomeric forms dominated by torsional motion at the C10–C11 bond. This suggests that the photochromic cycling of phytochromes between Pr and Pfr, which is known to be governed by $Z\rightarrow E$ (Pr→Pfr) and $E\rightarrow Z$ (Pfr→Pr) photoisomerizations at the C15–C16 bond,^{3–8,17–24} relies on interactions between the chromophore and the protein to prevent the chromophore from rather isomerizing at C10–C11. In particular, these findings indicate that the tight packing of pyrrole rings A–C shown by recent crystal structures,^{5–8} which contrasts the loose environment around ring D, plays an indispensable role for the photochromism of phytochromes by sterically hindering photoisomerizations at all other sites than C15–C16.

In an effort to better understand the source of the photochemical reactivity of the C10–C11 bond, we have performed additional calculations to assess the importance of intramolecular steric repulsion and charge distribution between the rings. From calculations on *ZaEaZs* PΦB, wherein the configuration of the BC bridge is changed from *Zs* (adopted by all chromophores in both Pr and Pfr) to *Ea*, it is

concluded that the steric repulsion between rings B and C present in the *Zs* configuration is not a contributing factor. From calculations comparing the FC relaxation of BV, PCB and PΦB in the absence and presence of a chloride anion, on the other hand, it is found that the greater positive charge of rings B and C^{45,56,58} is a major source for the photochemical reactivity of the C10–C11 bond. Furthermore, from this comparison it is also suggested that counterions in the vicinity of the A, B and C-ring nitrogens may facilitate the Pr→Lumi-R photoconversion by influencing the FC relaxation in such a way that the chromophore becomes less likely to isomerize at C10–C11 and more likely to isomerize at C15–C16.

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Table 1 Chromophore stereoisomers considered in the calculations

Bilin	Form	Stereoisomers		Experimental basis	Refs
BV	Pr	<i>ZsZsZa</i>		crystallography	5–7
BV	Pfr	<i>ZsZsEa</i>	<i>ZaZsEa</i>		
PCB	Pr	<i>ZsZsZa</i>		crystallography and NMR	8,9,28
PCB	Pfr	<i>ZsZsEa</i>	<i>ZaZsEa</i>		
PΦB	Pr	<i>ZaZsZa</i>		RR	26,27
PΦB	Pfr	<i>ZaZsEa</i>	<i>ZsZsEa</i>		

Table 2 Calculated absorption and fluorescence maxima of ZsZsZs DHBV

Level of theory ^a	Gas-phase absorption		Absorption in CH ₃ Cl		Gas-phase fluorescence		Fluorescence in CH ₃ Cl	
	eV	nm	eV	nm	eV	nm	eV	nm
B3LYP/SVP	2.13	582	2.05	605	1.70	729	1.63	761
B3LYP/TZVP	2.09	593	2.01	617	1.67	742	1.60	775
B3LYP/cc-pVDZ	2.13	582	2.05	605	1.69	734	1.63	761
B3LYP/aug-cc-pVDZ	2.08	596	2.00	620	1.66	747		
PBE0/SVP	2.18	569	2.10	590	1.74	713	1.67	742
M06-HF/SVP	2.53	490	2.45	506	2.03	611	1.95	636
BLYP/SVP	1.94	639	1.87	663	1.53	810	1.48	838
BP86/SVP	1.95	636	1.88	659	1.54	805	1.48	838
τHCTH/SVP	1.96	633	1.89	656	1.56	795	1.50	827
CAM-B3LYP/SVP	2.33	532	2.24	554	1.87	663	1.79	693
LC-ωPBE/SVP	2.49	498	2.41	515	2.02	614	1.93	642
Expt. ^b			2.10	590			1.80	690

^a All absorption maxima calculated using B3LYP/SVP S₀ geometries and all fluorescence maxima calculated using TD-B3LYP/SVP S₁ geometries. ^b Experimental data from ref 43.

Table 3 Optimized ground and excited-state bond lengths (Å) and dihedral angles (°) of BV, PCB and PΦB

Bilin		AB bridge				BC bridge				CD bridge			
		4–5		5–6		9–10		10–11		14–15		15–16	
		Bond	Angle	Bond	Angle	Bond	Angle	Bond	Angle	Bond	Angle	Bond	Angle
<i>ZsZsZa^a</i>	S ₀	1.370	-7.8	1.434	-15.0	1.406	8.9	1.393	8.2	1.420	171.8	1.381	-4.2
BV	S ₁	1.393	-11.8	1.412	-10.7	1.381	4.1	1.437	19.7	1.439	169.9	1.374	-5.1
<i>ZsZsEa^b</i>	S ₀	1.370	-8.0	1.436	-16.1	1.410	9.3	1.391	7.0	1.419	175.9	1.382	177.6
BV	S ₁	1.394	-13.0	1.414	-10.6	1.388	4.7	1.432	20.0	1.427	-178.8	1.383	170.9
<i>ZaZsEa^c</i>	S ₀	1.374	-3.9	1.427	168.6	1.401	10.9	1.395	9.8	1.424	170.1	1.380	176.1
BV	S ₁	1.393	-4.3	1.407	172.6	1.375	6.1	1.443	25.1	1.441	161.2	1.376	174.5
<i>ZsZsZa^a</i>	S ₀	1.367	-6.0	1.434	-17.0	1.399	9.3	1.398	10.7	1.425	170.4	1.376	-4.0
PCB	S ₁	1.383	-6.5	1.418	-13.3	1.382	4.8	1.444	26.1	1.431	169.2	1.384	-5.6
<i>ZsZsEa^b</i>	S ₀	1.366	-6.0	1.436	-17.7	1.402	9.5	1.396	10.0	1.425	173.0	1.377	176.8
PCB	S ₁	1.380	-7.0	1.427	-14.1	1.410	10.5	1.422	18.2	1.416	175.5	1.389	173.5
<i>ZaZsEa^c</i>	S ₀	1.372	-3.7	1.428	168.2	1.396	10.2	1.398	10.4	1.428	169.1	1.375	176.4
PCB	S ₁	1.380	-4.5	1.423	170.6	1.398	10.8	1.443	16.5	1.419	170.3	1.391	174.6
<i>ZaZsZa^a</i>	S ₀	1.372	-3.7	1.428	168.2	1.396	10.0	1.399	10.7	1.425	168.7	1.377	-4.2
PΦB	S ₁	1.379	-4.4	1.420	170.5	1.385	7.5	1.437	22.4	1.434	166.9	1.379	-3.6
<i>ZaZsEa^b</i>	S ₀	1.372	-3.8	1.428	168.0	1.397	10.2	1.398	10.3	1.426	169.0	1.378	176.1
PΦB	S ₁	1.380	-4.3	1.421	171.5	1.387	8.1	1.443	20.9	1.430	164.5	1.384	173.6
<i>ZsZsEa^c</i>	S ₀	1.365	-6.0	1.436	-17.7	1.402	9.5	1.396	9.9	1.423	172.7	1.379	176.5
PΦB	S ₁	1.380	-6.8	1.423	-13.3	1.390	6.5	1.434	21.1	1.429	175.1	1.383	172.8
<i>ZaEaZs</i>	S ₀	1.371	-3.7	1.428	170.5	1.396	-179.0	1.401	179.8	1.427	15.4	1.374	7.1
PΦB	S ₁	1.378	-4.7	1.424	169.8	1.395	-176.0	1.428	-174.0	1.425	14.6	1.383	7.3
bilin + Cl ⁻													
<i>ZsZsZa^a</i>	S ₀	1.379	-4.6	1.429	10.7	1.397	-1.4	1.404	2.4	1.432	-171.4	1.369	3.2
BV + Cl ⁻	S ₁	1.397	-5.5	1.412	8.2	1.395	-2.5	1.424	8.4	1.418	177.1	1.385	-2.4
<i>ZsZsZa^a</i>	S ₀	1.375	-5.5	1.430	12.5	1.393	-0.7	1.408	4.2	1.435	174.5	1.367	-1.8
PCB + Cl ⁻	S ₁	1.383	-4.2	1.428	10.6	1.415	-2.9	1.405	6.8	1.416	174.5	1.386	-4.5
<i>ZaZsZa^a</i>	S ₀	1.361	2.3	1.437	-172.3	1.396	-0.4	1.403	0.5	1.433	172.7	1.369	-2.6
PΦB + Cl ⁻	S ₁	1.371	2.7	1.433	-171.2	1.419	-0.1	1.401	0.6	1.413	174.6	1.390	-3.3

^a Geometry in Pr. ^b Geometry in Pfr if no thermal *syn/anti* isomerization during Pr→Pfr conversion. ^c Geometry in Pfr if thermal *syn/anti* isomerization at C5–C6 during Pr→Pfr conversion.

Table 4 Optimized ground and excited-state bond lengths (Å) and dihedral angles (°) of *ZaZsZa PΦB* at different levels of theory

Level of theory		AB bridge				BC bridge				CD bridge			
		4-5		5-6		9-10		10-11		14-15		15-16	
		Bond	Angle	Bond	Angle	Bond	Angle	Bond	Angle	Bond	Angle	Bond	Angle
B3LYP/SVP	S ₀	1.372	-3.7	1.428	168.2	1.396	10.0	1.399	10.7	1.425	168.7	1.377	-4.2
	S ₁	1.379	-4.4	1.420	170.5	1.385	7.5	1.437	22.4	1.434	166.9	1.379	-3.6
PBE0/SVP	S ₀	1.369	-4.0	1.424	166.8	1.392	10.3	1.396	11.2	1.422	167.2	1.373	-4.4
	S ₁	1.376	-4.4	1.417	169.9	1.388	8.4	1.433	22.0	1.420	168.1	1.382	-4.0
B3LYP/TZVP	S ₀	1.363	-3.8	1.422	167.1	1.388	9.9	1.392	10.6	1.419	167.9	1.368	-3.9
	S ₁	1.371	-4.4	1.413	170.0	1.374	7.1	1.434	24.4	1.427	167.2	1.371	-3.4

Figure captions

Fig. 1 Chemical structures of the BV (biliverdin IX α), PCB (phycocyanobilin) and P Φ B (phytochromobilin) chromophores of bacterial, cyanobacterial and plant phytochromes shown in the C5-*Z*,*syn* C10-*Z*,*syn* C15-*Z*,*anti* (*ZsZsZa*) configuration. Each chromophore forms (at C3) a thioether linkage with a cysteine residue of the apoprotein.

Fig. 2 Chemical structure of the *ZsZsZs* dihydrobiliverdin species used for testing the accuracy of the computational approach.

Fig. 3 Optimized excited-state structures of BV, PCB and P Φ B. Numerical values indicate changes in dihedral angles ($^{\circ}$) relative the ground state.

Fig. 4 S_1 potential energy curves for the initial stages of photoisomerizations of *ZsZsZa* BV and *ZsZsEa* BV at the C4–C5, C10–C11 and C15–C16 bonds. Energies (E) are given relative the fully optimized excited-state minima.

Fig. 5 Optimized excited-state structure of *ZaEaZs* P Φ B. Numerical values indicate changes in dihedral angles ($^{\circ}$) relative the ground state.

Fig. 6 (a) Bond length differences between the S_1 and S_0 states of *ZsZsZa* BV, *ZsZsZa* PCB and *ZaZsZa* P Φ B. (b) Same data with a chloride anion present.

Resonance structures of the
tetrapyrrolic skeleton of BV

Fig. 1

Fig. 2

BV

ZsZsZa

ZsZsEa

ZaZsEa

PCB

ZsZsZa

ZsZsEa

ZaZsEa

PΦB

ZaZsZa

ZaZsEa

ZsZsEa

Geometry in Pr

Geometry in Pfr if no thermal *syn/anti* isomerization during Pr→Pfr conversion

Geometry in Pfr if thermal *syn/anti* isomerization at C5–C6 during Pr→Pfr conversion

Fig. 3

Fig. 4

Fig. 5

Fig. 6