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ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · JANUARY 2009

Impact Factor: 3.3 · DOI: 10.1021/jp807471e · Source: PubMed

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The Chromophore Structure of the Cyanobacterial Phytochrome Cph1 As Predicted by Time-Dependent Density Functional Theory

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Received: August 20, 2008; Revised Manuscript Received: September 25, 2008

The UV−vis absorption spectra of the photoreceptor chromophores biliverdin (BV) in the ZZZssa conformation and the phycocyanobilin (PCB) with conformations ZZZssa and ZZZasa have been investigated by means of time-dependent density functional theory (TD-DFT) with a polarized continuum model. The three systems are studied in different conditions to include protonation, solvation- and protein-environmental effects on gas phase and available X-ray structures. The crystal structures of BV in bacteriophytochrome of *Deinococcus radiodurans* and PCB in C-Phycocyanin serve to calibrate the performance of the TD-DFT method and allow estimating the spectral shifts created when gas phase structures instead of a proper environment are used. In contrast, the structure of PCB in the cyanobacterial phytochrome Cph1 is unknown. The excellent agreement of the theoretical spectrum with experimentally recorded data for the PCB in the cyanobacterial phytochrome Cph1 strongly supports a semicyclic ZZZssa structure, similar to that found for the BV chromophore.

Phytochromes are a family of photoreceptors present in all flowering plants (Phy family) and cryptophytes, but also in cyanobacteria (phytochromes Cph1 and Cph2), nonoxygenic bacteria (bacteriophytochromes or BphPs), and even fungi (fungal phytochromes or Fphs). Each photoreceptor has a chromophore whose function depends on the nature of the phytochrome. In most plant phytochromes the chromophore is the phytochromobilin (PΦB), while phycocyanobilin (PCB) is in general related to cyanobacteria and biliverdin (BV) to nonoxygenic bacteria.[1] All these chromophores are bilins and show a covalently linked open-chain tetrapyrrole (see Figure 1), which is structurally related with the macrocyclic tetrapyrrole structures of the well-known porphyrins. As such, they show a similar absorption spectrum, governed by the so-called Soret and Q bands.[2] In the visible absorption spectra of porphyrins the intense absorption band located between 350 and 450 nm is known as the Soret band and the typically weak absorption bands between 450 and 700 nm are called Q bands.[3,4] Likewise, bilins show a Soret band in the visible absorption region but only one Q-band. The chromophore of plant phytochromes controls many photomorphogenic processes regulating the metabolic response of the organism to its light environment. Upon light irradiation, phytochromes can switch from the inactive P_r, red-absorbing (R) form with a Q-band peaking around 666 nm, to the active P_{fr}, far-red (FR) absorbing form with a peak around 730 nm.[5] This interconversion is light reversible and it is well known[6] that the difference between P_r and P_{fr} involves a Z-E photoisomerization at the double bond C15═C16, (see Figure 1).

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While numerous spectra have been available for phytochromes for several decades, crystal structures are rather scarce. Only a few proteins have been resolved in the last several years, namely, the one corresponding to the chromophore-binding domain of the bacterial phytochrome of *Deinococcus radiodurans* (*Dr*BphP) with a BV chromophore in the P_r state[7,8] and the chromophore-binding domain of an unusual bacterial phytochrome RpBphP3 from *Rhodompseudomonas palustris*, also with a BV chromophore.[9] In the X-ray structure of *Dr*BphP, the P_r form of BV adopts a semicyclic ZZZssa (C_5-Z, C_{10}-Z, C_{15}-Z, C_5-syn, C_{10}-syn, C_{15}-anti) conformation (see Figure 1) and is covalently attached to a cysteine residue near the N-terminal domain by a thioether bond.[7] Unfortunately, the crystal structure of the cyanobacterial phytochrome has not yet been obtained. Thus, very often theoretical calculations or mechanistic studies on phytochromes are done using the PCB chromophore of the C-phycocyanin (C-PC), which is a light-harvesting pigment present in photosynthetic cyanobacteria for which the crystal structure was resolved long ago.[10] In contrast to the semicyclic structure of BV, PCB in C-PC shows an extended ZZZasa conformation.

Nowadays, there is no consensus about the paramount question of chromophore conformation in cyanobacterial and plant phytochromes.[1,11,12] On one hand, a ZZZssa structure, as in the BV chromophore of *Dr*BphP, is put forward for the P_r forms of PCB and PΦB with arguments such as the high sequence identity between bacterial, cyanobacterial, and plant phytochromes.[1,11] Furthermore, two-dimensional nuclear Overhauser effect[13] as well as ^{15}N NMR spectroscopy[14] experiments are consistent with a ZZZssa conformation for cyanobacterial phytochrome Cph1. On the other hand, theoretical and experimental resonance Raman (RR) spectra suggest a ZZZasa

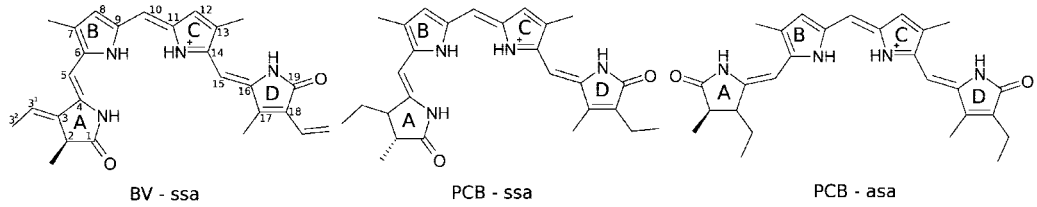

Figure 1. Protonated biliverdin (BV) and phycocyanin (PCB) chromophores with conformations as indicated. Propionic side chains in rings B and C are not considered.

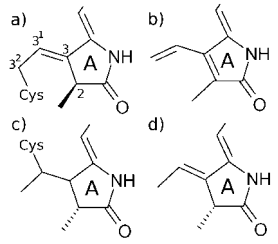

Figure 2. Possible conformations which can be considered for the ring A of the chromophores BV (a and b) and PCB (c and d). In panels a and c, the chromophores are assembled to the apoprotein, while in panels b an d they are not.

structure.[12,15,16] In the present study, we shed light on this conformational controversy through quantum chemical calculations on PCB in Cph1. First, we investigate the UV−vis absorption spectra of the chromophores BV in BphP and PCB in C-PC for which three-dimensional structures are available. Then we use different models to probe the spectra of PCB in Cph1, and we discuss the structure which would be consistent with the available experimental data.

The calculations are done on the three structures shown in Figure 1. As template the crystal structure of the bacteriophytochrome chromophore binding domain at 1.45 Å resolution (PDB: 2O9C) is used for the BV and PCB chromophores in the ZZZssa conformation,[7] while the X-ray structure at 1.45 Å resolution of the α-84 subunit of C-PC from the thermophilic cyanobacterium *Synechococcus elongatus* (PDB: 1JBO) is used for PCB in the ZZZasa conformation.[17] The hydrogen atoms are added according to the molecular arrangement of BV and PCB after the assembly with the apoprotein (see Figure 2 and later in text). In all cases the cysteine linkage was replaced with hydrogen. The propionic-acid sidechains on the rings B and C (cf. Figure 1) are not included because they are not part of the conjugated system of the chromophore and as such they do not affect the excitations energies (data not shown), which is in agreement with previous studies.[18] For each system, four models of different complexity have been considered to account for protonation, solvation, and protein surroundings. Model I is the unprotonated chromphore in vacuo. Model II is the protonated form also in vacuo. Model III and IV are protonated forms simulated in water and in a protein environment, respectively. The spectra is calculated not only on X-ray structures, but also on chromophores previously optimized using density functional theory DFT with the B3LYP/6-31G(d) protocol,[19] as implemented in the Gaussian03 set of programs.[20] A calculation of the Hessian ensured that the obtained geometries are true minima, which can be found in the Supporting Information. Vertical excited states and corresponding oscillator strengths are obtained using the time-dependent[21] version of B3LYP/6-31G(d) over eight roots. The so-obtained spectra are then convoluted with Gaussian functions with full widths of 4000 cm^{-1} at half-maximum using the GaussSum 2.1 program.[22] The environment is modeled with the polarizable continuum model (PCM).[23] A dielectric constant of $\varepsilon = 78.4$ is used for water, and $\varepsilon = 4.0$ is used to represent the surrounding protein moiety, as first suggested by Blomberg et al.[24] and later on by others.[25−27]

Experimentally, the absorption spectra of BV in BphP,[8,28] PCB in Cph1,[29] and PCB in C-PC[10] show a UV Soret band centered in all cases at 380 nm and a Q-band around 700 nm. Since the exact position of the Q-band depends on the specific chromophore and protein, the accuracy describing this part of the spectrum will be used as a criterion to discern a semicyclic (ssa) from a extended (asa) conformation of the unknown PCB phytochrome in Cph1. Table 1 collects the experimental absorption Q peaks recorded for the plant (oat) and cyanobacteria chromophores, as well as the calculated values for the Models I−IV in both the X-ray and DFT optimized structures. Other theoretical values from the literature are also compiled in Table 1.

First we compare the absorption peaks calculated on the relaxed and on the X-ray geometries with each other. We can see that the Q bands are rather different, giving account of the steric constraints imposed by the protein moiety. Specific observed changes are that the optimized structures are more closed (or cyclic) than the X-ray ones and the rings A, B, and C show deviations from planarity. As a consequence, every relaxed structure, regardless of the conditions I−IV, exhibits a markedly blue shift with respect to the crystal structure and the

TABLE 1: TD-B3LYP/6-31G(d) Q-bands (in nm) Contributing to the UV−Vis Absorption Spectra of the Chromophores BV and PCB Calculated on X-ray Structures and DFT Optimized Ones in Different Conformations in Different Environments; Experimental and Other DFT Values Are Given for Comparison

		model I (unprotonated/in vacuo)	model II (protonated/in vacuo)	model III (protonated/water)	model IV (protonated/protein)	experimental
BV-ssa	X-ray	648	662	708	712	702 (BV in BphP)[a]
	DFT	588	643	659	665	
PCB-ssa	X-ray	603	620	659	661	659 (PCB in Cph1)[b]
	DFT	526	590	609	613	
	DFT		574[d]			
PCB-asa	X-ray	528	590	606	614	618 (PCB in C-PC)[c]
	DFT	508	559	584	588	
	DFT	539[e]	541[d], 582[e]			

[a] References 8 and 28. [b] Reference 29. [c] Reference 10. [d] Reference 13. [e] Reference 25.

experimental values. The difference between the DFT results from the literature[13,25] and our values is related to the structure of the ring A (Figure 2) adopted in the calculations. This aspect can be fiddly since the structure of the A ring depends, among other reasons, on whether the chromophore is assembled or not to the apoprotein. Our calculations for BV follow the most recent X-ray structure resolved in 2007 which undoubtedly revealed a chiral center at the carbon C2 after ligation of the cysteine residue to the $C3^2$ carbon[8] (Figure 2a). Caution should be exercised in not considering the unassembled structure (Figure 2b), since this contains one double bond more in the conjugated π system, which typically results in a red shift of 30 nm.[8] Analogously to BV, for PCB we have used the structure shown in Figure 2c. In contrast, the calculations of Wan and co-workers[25] adopt the chromophore before the apoprotein is linked via the cysteine residue at the $C3^1$ carbon (see Figure 2d); this structure implies an additional $C3{=}C3^1$ double bond, with the concomitant red shift of ca. 30 nm (compare 508 and 559 nm with 539 and 582 nm, respectively, in Table 1). The explanation for the difference between the Q peaks obtained by van Thor[13] et al. and ours is more subtle. They used the appropriate assembled chromophores for BV and PCB (Figure 2a,c, respectively), but a different density functional (MPW1PW91), leading to a blue shift of ca. 15−20 nm with respect to our B3LYP values (see Table 1).

From all these considerations and our results, we conclude that calculated spectra based on relaxed geometries should be treated with caution, and henceforth we shall only discuss the changes on the spectra calculated on the crystal models.

Since the crystal structure cannot evidence protonation, it is important to evaluate its effect on the absorption spectra. As it can be seen, upon protonation the three chromophores suffer a bathocromic shift; taking this into account, the neutral form shows a larger deviation from the experimental values. The difference between the neutral and the protonated forms is about 15 nm for the conformation ZZZssa and larger for the ZZZasa conformation with a difference of more than 60 nm. Hence, our calculations support the fact that the chromophores are protonated in agreement with refs 25 and 30−34.

Aqueous solution induces a variable solvatochromic red shift in all chromophores. Conspicuously, a similar effect is obtained in the protein environment. These values come very close to the experimental ones with an accuracy seldom achieved with TD-DFT. The experimental values of 702 nm for BV-ssa and 618 nm for PCB-asa in C-PC are quantitatively reproduced by the theoretical 708/712 nm and 606/614 nm ones, respectively (see Table 1). We now turn to PCB in Cph1. Since the calculated peaks at 659 and 661 nm for water and protein environments are in excellent agreement with the experimental Q-band measured at 659 nm and far away from 618 nm, which would correspond to a ZZZasa conformation, we are left to conclude that PCB in Cph1 must adopt a ZZZssa conformation. It is also gratifying to realize that our calculations are also consistent with a hypsochromic shift which takes place when going to more extended conformations (in this case from ssa to asa), as it has been observed with semiempirical AM1 calculations on the PCB system.[35]

To uncover more details of the UV−vis spectra of the three chromophores, we show in Figure 3 the calculated absorption bands in protein media, which we consider the most accurate ones. As it can be seen the Soret band comprises several states with different oscillator strengths. In contrast, the Q-band is described by a single state which upon inspection of the contributing orbitals can be attributed to an highest occupied molecular orbital (HOMO)→lowest

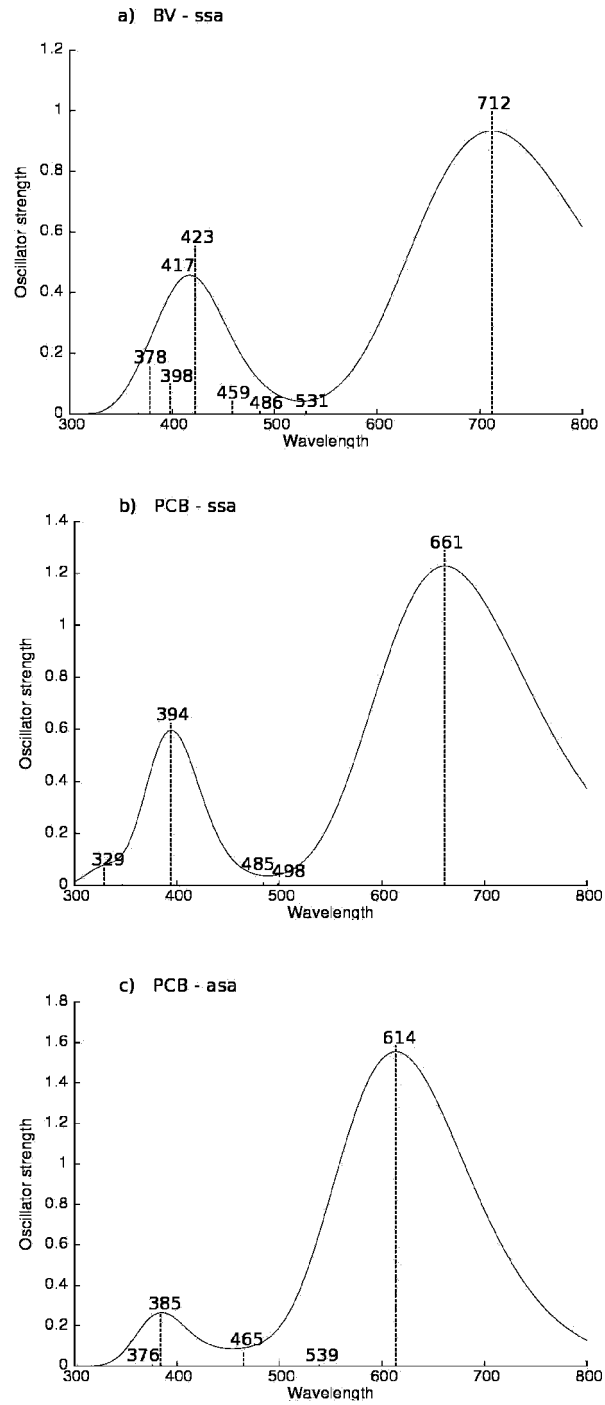

Figure 3. TD-DFT absorption UV−vis spectra of the protonated BV-ssa (a), PCB-ssa (b), and PCB-asa (c) chromophores modeled in a protein environment ($e = 4$).

unoccupied molecular orbital (LUMO) transition in the three chromophores. The strongest peak contributing to the Soret band is a state with a superposition of HOMO-1→LUMO and HOMO→LUMO+1 excitations. The corresponding orbitals for PCB-ssa are shown in Figure 4; all of them correspond to π,π^* orbitals delocalized between the four pyrrole rings.

In conclusion, the calculated spectra suggest that the P_r form of PCB very likely adopts a ZZZssa conformation in the cyanobacterial phytochrome. This conclusion fits into the picture proposed by Lagarias et al. who analyzed a sequence alignment of 122 known (or suspected) phytochromes and phytochrome-related proteins finding that (i) all phytochromes exhibit sequence conservation in two of the three domains of the

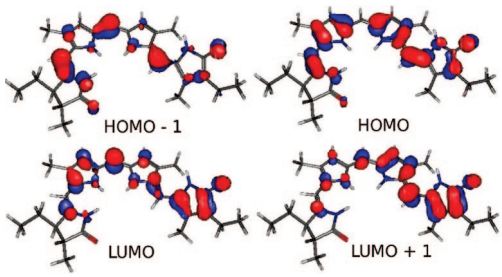

Figure 4. Selected molecular orbitals of PCB-ssa involved in the absorption spectra given in Figure 2b.

photosensory region, (ii) key residues in the knot between these two domains are also conserved, and (iii) differences in the sequences are not within the secondary structure.[1,11] These facts indicate that the architecture of the photosensory core is very likely to be conserved in all phytochromes, implying then that the chromophores should all have the same ZZZssa conformation as found in bacteriophytochromes. This proposal is backed up by the recent ^{13}C- and ^{15}N NMR spectroscopic experiments of refs 13 and 14 respectively, performed in the cyanobacterial phytochrome Cph1. Moreover, taking into account that in both plants and cyanobacteria the chromophore links in the same way to the apoprotein (by a cysteine at the 3^1 carbon, see Figure 1), it is very plausible that the plant phytochrome PΦB also presents a ZZZssa conformation. Lagarias and co-workers have gone even further on proposing a photoconversion mechanism which involves a semicyclic ssa conformation for the P_{fr} form too, with no net charge transport over the full path.[1]

Contrary to the appealing idea of a unified ssa conformation for all the phytochrome species, the RR data of Mroginski et al.[15,11] suggest an asa structure for both the P_r and P_{fr} states, as well as for the intermediate Lumi-R of the photocycle. However, one should note that these calculations have been done in vacuo, that is, without the protein environment, even when it has been demonstrated that both the experimental[36] and theoretical[37] RR spectra are very sensitive to the protein environment. Indeed, the recent hybrid quantum mechanics/molecular mechanics (QM/MM) study on the PCB chromophore explicitly bound to the α-subunit of C-PC clearly shows significant improvements with respect to the pure QM calculations of the isolated chromophore indicating that the comparison of experimental RR spectra of the protein-bound chromophore with calculated RR spectra of the isolated cofactor may not always be unambiguous.[37]

Summarizing, according to our results protonation and the conformational change from asa to ssa induce a bathochromic shift, whereas deprotonation and the isomerization from ssa to asa induce a hypsochromic shift. Most importantly, the excellent agreement between the experimental absorption spectra and the herein calculated one provides additional support that the Cph1 phytochrome adopts a ZZZssa conformation as in the bacteriophytochrome.

Acknowledgment. We thank early discussions with Dr. M.A. Mroginski (TU Berlin) and financial support from the Deutsche Forschungsgemeinschaft (GO 1059/2-1) as well as CONICYT (Chile) and DAAD (Germany).

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added in Proof. At the time of processing this article, a ZZZssa confirmation was found in two cyanobacterial chromophores by NMR[38] and diffraction[39] experiments.

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