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MAS NMR study of the photoreceptor phytochrome

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Chapter 5

Discussion and outlook

In photoreceptors, the properties of the chromophore are influenced by the protein matrix. For instance, opsin contains the retinylidene cofactor in an 11-*cis*-12-*s-trans* conformation. MAS NMR spectroscopy has provided valuable information about the interaction between the retinylidene cofactor and its binding pocket in rhodopsin [125–127] by demonstrating that the non-bonding interaction between the C13 methyl group and the hydrogen of C10 give rise to a non-planar structure. The resulting torsions in the chromophore pre-configure the polyene for the photoisomerization occurring at the C11=C12 double bond [127–130]. A similar effect can be found for the phytochromes: PCB in its free state adopts a different conformation in solution (*ZZZsss*) than in its protein-bound state (*ZZZssa*), and the stabilization of the Pfr form (*ZZEssa*) is only accomplished by the protein.

PCB in its free state has been studied in **Chapter 2** and as chromophore in phytochrome in both **Chapter 3** and **Chapter 4**. Almost complete sets of ^{13}C and ^{15}N chemical shifts have been obtained for the free PCB and for the chromophore in the Pfr, Lumi-F, Meta-F and Pr states. In this chapter, the ^{13}C chemical shifts of PCB as free open-chain tetrapyrroles and chromophore in phytochrome are compared to elucidate the effect of the protein pocket.

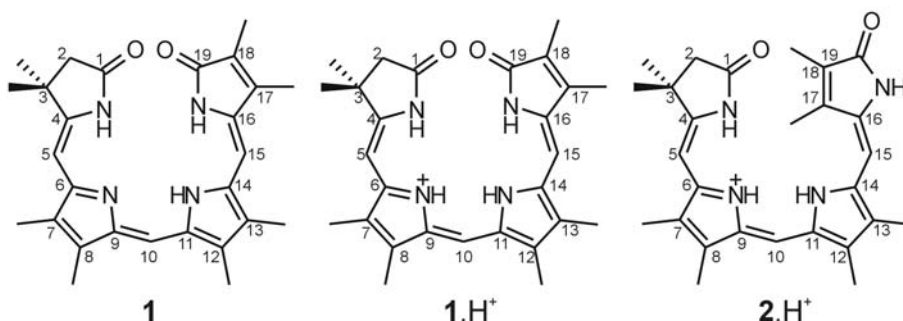


Figure 5.1: Structures of the **1** and **1.H⁺** model compounds in the *ZZZ_{ss}* geometry. **2.H⁺** is the *E*-diastereoisomer of **1.H⁺** in its *ZZE_{ss}* geometry. The extent of positive charge is respresented on ring **B**, as assumed in [103].

5.1 Comparison with model compounds

5.1.1 Effect of protein environment on PCB in phytochrome

In the late 1990's, Stanek and Grubmayr [103] have investigated the 2,3-dihydro-23*H*-bilin-1,19(21*H*,24*H*)-dione molecules **1**, **1.H⁺** and **2.H⁺** (Fig. 5.1). These three compounds are member of the 2,3-dihydrobilindione family. The compound **1** and its protonated form **1.H⁺** have been shown to adopt a helical shape with a *ZZZ_{ss}* geometry in CDCl₃ solution. The *E*-diastereoisomer of **1.H⁺**, **2.H⁺**, also occurs in a helical structure with a *ZZE_{ss}* geometry. By comparing the chemical shift values of the carbons positioned symmetrically within the dipyrin formed by rings **B** and **C** (C6 and C14, 152.4 *vs.* 142.1 ppm, respectively), Stanek and Grubmayr concluded that an extent of positive charge is located on ring **B** in **1.H⁺**.

The change in ¹³C chemical shift after the binding of PCB in the protein pocket in the Pr state is shown in Figure 5.2A. The effect observed at the ring **A**, especially at the C3, C3¹, C3² and C4 (-82.8, -77.4, +5.7 and +7.7 ppm, respectively, Table 5.1) are in line with the formation of the thioether linkage with Cys-259 at C3¹.

The modification induced by the protonation of N22 occurring during the assembly of PCB in the protein pocket are visible at C6, C9, C12, C14 and C16. The effects of the protonation of N22 on the ¹³C chemical shifts in the model compound **1** are depicted in Figure 5.2B. Comparing Figures 5.2A and B, the overall patterns of the change in ¹³C chemical shift are similar. C6 and

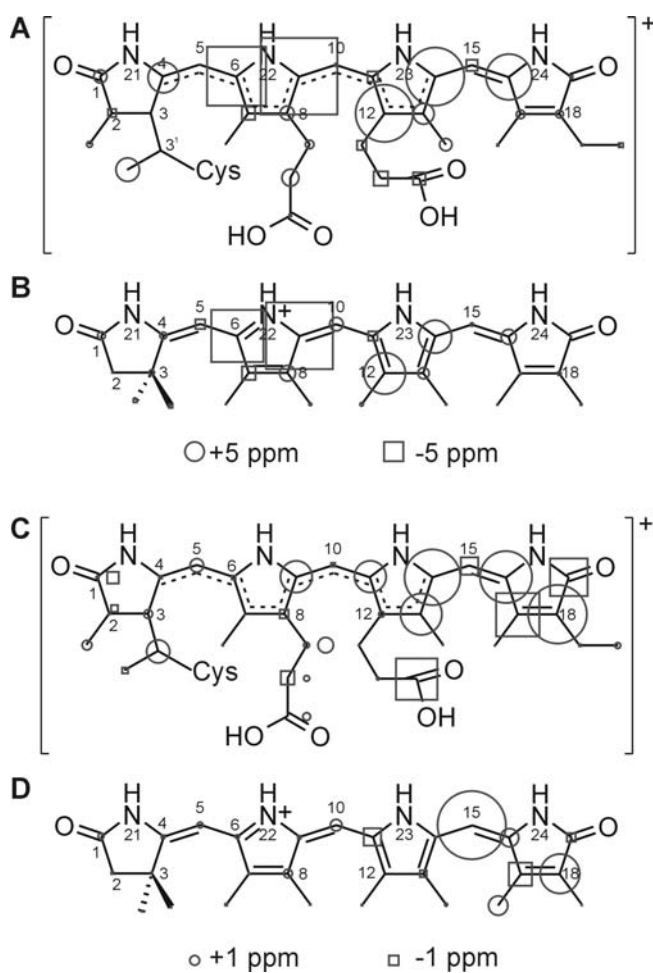


Figure 5.2: The upper part (A and B) shows the change in ^{13}C chemical shifts resulting from the assembly of PCB into Cph1Δ2 (PCB $\rightarrow$ Pr, A) and upon protonation of 1 (1 $\rightarrow$ 1.H $^+$, B). The lower part (C and D) represents the changes in ^{13}C chemical shifts accompanying Pr $\rightarrow$ Pfr conversion (C) and the difference in ^{13}C shifts between the models compound of the 1.H $^+$ and 2.H $^+$ (D). The free PCB (A form), 1, Pr and 1.H $^+$ are taken as reference in A, B, C and D, respectively. The upshifts of the ^{13}C resonances of C3 and C3 1 due to the formation of the thioether linkage with Cys-259 (82.8 and 77.4, respectively) are not represented in A.

Position	1	1.H⁺	1 $\rightarrow$ 1.H⁺	PCB	Pr	PCB $\rightarrow$ Pr
C1	175.9	177.9	2.0	179.4	182.5	3.1
C2	44.2	44.0	-0.2	39.3	37.1	-2.2
C3	39.5	40.9	1.4	136.2	53.4	-82.8
C3 ¹	29.4	28.5	-0.9	125.2	47.6	-77.4
C3 ²	—	—	—	16.1	21.8	5.7
C4	159.3	161.1	1.8	146.2	153.9	7.7
C5	89.9	87.4	-2.5	87.0	87.1	0.1
C6	166.0	152.4	-13.6	164.7	149.1	-15.6
C7	131.0	127.5	-3.5	129.5	126.1	-3.4
C7 ¹	9.9	9.8	-0.1	9.3	9.2	-0.1
C8	139.5	143.6	4.1	142.1	144.5	2.4
C8 ¹	9.9	10.6	0.7	22.9	22.5	-0.4
C8 ²	—	—	—	36.8	43.1	6.3
C8 ³	—	—	—	180.0	180.5	0.5
C9	149.4	132.0	-17.4	147.8	127.7	-20.1
C10	111.3	114.8	3.5	112.2	112.7	0.5
C11	132.7	130.3	-2.4	130.6	127.7	-2.9
C12	128.6	139.4	10.8	130.8	145.5	14.7
C12 ¹	9.5	10.4	0.9	20.4	21.1	0.7
C12 ²	—	—	—	42.4	37.5	-4.9
C12 ³	—	—	—	182.2	179.5	-2.7
C13	124.2	127.1	2.9	121.3	126.3	5.0
C13 ¹	9.4	9.8	0.4	8.4	11.3	2.9
C14	133.5	142.1	8.6	131.1	145.7	14.6
C15	96.6	97.5	0.9	96.0	93.5	-2.5
C16	136.3	140.2	3.9	134.3	145.7	11.4
C17	141.7	142.1	0.4	140.1	142.2	2.1
C17 ¹	10.0	9.8	-0.2	10.1	9.8	-0.3
C18	127.6	128.6	1.0	132.0	134.3	2.3
C18 ¹	8.7	8.2	-0.5	16.8	16.1	-0.7
C18 ²	—	—	—	14.3	13.3	-1.0
C19	173.6	173.5	-0.1	173.7	172.8	-0.9

Table 5.1: ¹³C chemical shifts of **1** and **1.H⁺** are taken from [103]. PCB (A form) and Pr have been studied in **Chapter 2** and **Chapter 3**, respectively.

C9 are respectively upshifted by 13.6 and 17.4 ppm in the model compound, while the upshifts of these resonances are of 15.6 and 20.1 ppm upon assembly in the protein (Table 5.1). These strong changes can be directly related to the protonation on N22. The significant downshifts at C12 and C14 are also observed in both Figures 5.2A and B, indicating that the protonation of N22 induces a modification of the electronic structure of the tetrapyrroles moieties. The downshifts at C12 and C14 are markedly larger upon assembly of PCB in the protein (14.7 and 14.6 ppm, respectively) than in the model compound (10.8 and 8.6 ppm, respectively). The more pronounced up- and downshift of the ¹³C chemical shifts in the protein suggest that the protein pocket has an

influence on the positive charge delocalization over the rings **B** and **C** in the Pr state.

On the other hand, despite the overall similarity of the pattern, the changes in the ^{13}C resonances along the C15 methine bridge are significantly stronger in the case of the PCB assembly in the protein compared to the protonation of the model compound. Hence, these differences indicate an additional effect of the protein environment at these positions. Indeed, the PCB molecule adopts a *ZZZsss* geometry in its free state (**Chapter 2** and [24]), while the X-ray structure in the Pr state revealed a *ZZZssa* geometry in the protein [53]. Therefore, the *syn*-to-*anti* conformational change of the C14-C15 single bond appears to be caused by the protein inducing a dihedral angle of 26.3° between the planes formed by rings **C**-**D** [53]. In the X-ray structure of Cph1 Δ 2, the *anti* conformation appears to be stabilized by the hydrogen-bonding interaction between the C19 carbonyl and N24 with His-290. In addition, the methyl substituent at C17 is close to the Tyr-263 hydroxyl (2.66 Å). Apparently, this steric interaction prevents a more planar conformation between rings **C**-**D**. In this geometry, the C13¹ methyl group is in close contact with N24 (2.84 Å). It can be assumed that this steric interaction is responsible for the enhanced downshift of the C13 and C13¹ resonances. It cannot, however, currently be decided whether these effects around the C15 methine bond are caused solely by steric or also electronic effects.

5.1.2 Pr $\rightarrow$ Pfr conversion

The changes in ^{13}C chemical shifts caused by the Pr $\rightarrow$ Pfr conversion in Cph1 Δ 2 (Fig. 5.2C) have been analyzed in **Chapter 3**. The changes along the C15=C16 double bond and in the entire ring **D** in Cph1 Δ 2 have been related to the formation of the strong hydrogen-bonding interaction of the C19 carbonyl in the Pfr state, which lowers the bond order of the C15=C16 and the C17=C18 bonds and to a potential redistribution of the positive charge.

The differences in the ^{13}C resonances between **1.H**⁺ and its *E*-diastereoisomer **2.H**⁺ are depicted in Figure 5.2D. The patterns of the Figures 5.2C and D, in particularly along the C15 methine bridge, are significantly different. The alternating up- and downshift pattern is inverted from C8 to C15. The C14, C15 and C16 are shifted by +5.4, -1.8 and +5.0 ppm in Cph1 Δ 2, respectively, while the corresponding differences between **1.H**⁺ and **2.H**⁺ are

of -0.1, +7.1 and +1.9 ppm (Table 5.1). Although the alternating up- and downshifts of the ^{13}C resonances between C16 and C19 have the same phase, they are remarkably stronger for the protein-bound case and may indicate an increase of charge density alternation between the odd and even numbered carbon atoms at these positions.

Obviously, the double-bond isomerizations in both systems are of fundamentally different characters. For the isomerization in phytochrome, ring **C** has been proposed to be the hot spot for the change of the electronic structure, involving a potential redistribution of the positive charge along the chromophore (**Chapter 3**). The double bond isomerization of the model compound is expected to occur in a classical way, *i.e.* a rotation of 180° , as for example in the well-investigated stilbene [131]. In addition, the C14–C15 single bonds of the **1.H**⁺ and **2.H**⁺ model compounds have a *syn* conformation in solution, while this single bond forced into an *anti* conformation in both Pr and Pfr states [18,51–53,55]. The *anti* conformation of the C14–C15 in phytochrome induces an intramolecular interaction between the C13¹ and C17¹ methyl groups and may prevent planarity of the rings **C** and **D** in the Pfr state. The pattern of the change in ^{13}C chemical shift at the C15 methine bridge in Cph1Δ2 may originate from the contribution of three effects: steric interaction, hydrogen-bonding interaction and extended delocalization of the positive charge in the Pfr state.

5.1.3 Photoisomerization in phytochrome

In the free state, open-chain tetrapyrroles commonly adopt a helical shape with a *ZZZsss* geometry, as it has been shown for the biliverdin dimethyl ester-IXα in solution [132, 133] as well as in its crystalline state [85]. Upon irradiation with white light, the photochemistry of the biliverdin dimethyl ester IXα leads to *Z*-to-*E* photoisomerizations at one of the two outer methine bridge, *i.e.*, at C4=C5 and C15=C16, while no photoisomerization has been observed at the central C10 methine bridge [132, 134]. On the other hand, time-dependent DFT calculations suggest that an isolated protonated PCB moiety forms photochemically preferably C10-methine isomers [135].

In phytochrome, the photoisomerization is generally accepted to occur selectively at the C15=C16 double bond of the chromophore. The NMR data obtained in this thesis are in agreement with this assumption. Since the intrinsic reactivity of the free protonated chromophore may favor a photoreaction

at C10, the localization of the photoisomerization in phytochrome may be a result of chromophore/protein interactions, which modulate and tune the photochemical properties of the bound chromophore. All crystal structures of phytochromes show that PCB binds to the protein with *Z*, *anti* geometry at the C15 methine bridge. The rings **A**, **B** and **C** are tightly packed into the protein pocket. The steric interactions, for example by His-260, as well as the hydrogen-bonding interactions fix the structure in a state in which rings **A**, **B** and **C** are almost coplanar, and may thus prevent photoisomerization at the C5 and C10 methine bridges. This is in line with the moderate chemical shift changes observed at these positions (Fig. 5.2C). On the other hand, ring **D** is rotated by an angle of about 25 to 45° [18,51–53] and resides in a cavity that allows for relatively unhindered rotation around the C15 methine bridge. The relative freedom of ring **D** and the torsion around the C14–C15 bond may direct the photoisomerization at the methine bridge between rings **C** and **D**.

5.2 Results and prospects

The experimental results obtained in this thesis have demonstrated that the combination of isotope labeling and solid-state NMR spectroscopy is a powerful tool for the investigation of the photochemical switching machinery of phytochrome. The ^1H , ^{15}N and ^{13}C assignments provided information at the atomic level about the specific chromophore/protein interactions within the chromophore binding domain in the Pfr, Lumi-F, Meta-F and Pr states. The ^{13}C chemical shift data of the chromophore allow for determination of the electronic structure of the chromophore in these states. Due to selective isotope labeling of N21 and the study of the free PCB, one of the four ^{15}N signals, N21, has been assigned, and a tentative assignment of N22, N23 and N24 has been proposed for all studied states.

In **Chapter 3**, it has been shown that the chromophore is stabilized by hydrogen-bonding interaction at the C19 carbonyl group. The increase in length and strength of the conjugation of the π -system implies a strong hydrogen-bonding interaction at the carbonyl of ring **D**, causing the red shift of the maximum of absorption in the Pfr state. It has been proposed that Tyr-263 acts as hydrogen-bonding partner of C19.

MAS NMR provided new insights into the mechanism of the back-reaction.

The ability to produce samples in the pure Pfr state combined with low-temperature MAS NMR and an illumination setup allowed for trapping and studying of the two intermediates of the back-reaction, Lumi-F and Meta-F (**Chapter 4**). It has been shown that N24 also contributes to the stabilization of the chromophore in the Pfr state *via* hydrogen-bonding interaction with Asp-207. The back-reaction process begins with the photoisomerization of the C15=C16 double bond, which is followed by the rupture of the hydrogen-bonding interaction of N24, enabling the rotation of the C14-C15 single bond to take place. The method presented here for the study of the Pfr $\rightarrow$ Pr conversion may also be applied to the investigation of the intermediates present in the pathway from Pr $\rightarrow$ Pfr.

The ^{13}C and ^{15}N chemical shifts presented in **Chapter 3** and **Chapter 4** are the ideal starting point for theoretical studies, which may probe the proposed increase in conjugation of the π -system in the Pfr state. In addition, DFT calculations may bring further insights about the location of the positive charge within the chromophore as well as the potential redistribution of this charge during the photocycle.

The ^{13}C and ^{15}N assignments in the Pfr, Lumi-F, Meta-F and Pr states presented in this thesis will help to design further MAS NMR experiments, which are required to elucidate the changes of the hydrogen-bonding network within the chromophore binding site. In particular, MeLoDi-type of long-distance ^1H - ^{13}C or ^1H - ^{15}N heteronuclear correlation MAS NMR experiments and selective labeling of individual amino acids will help to investigate the complex cofactor/protein interactions [126, 136]. In addition, the measurement of internuclear distances [137] and angles [138] by MAS NMR would provide the structural information required for the full description of the rotation of ring **D** and allow for probing of the behavior of the C5 and C10 methine bridges.

Due to the similarity of the NMR spectra of Cph1 Δ 2 and *phyA*65 in the two parent states Pr and Pfr (**Chapter 3**), most of the interactions occurring in Cph1 Δ 2 are believed to be also present in plant Phys. However, small but distinct differences in ^{13}C shift are observed, especially at the methine bridge between ring **C** and **D**. The 1D ^{15}N spectra of the Cph1 Δ 2 and *phyA*65 assembled to an uniformly ^{15}N labeled PCB chromophore in the Pfr, Meta-F and Pr states are presented in Figure 5.3. In analogy to the ^{13}C NMR spectra, the pattern of the ^{15}N spectra of Cph1 Δ 2 and *phyA*65 are similar. However,

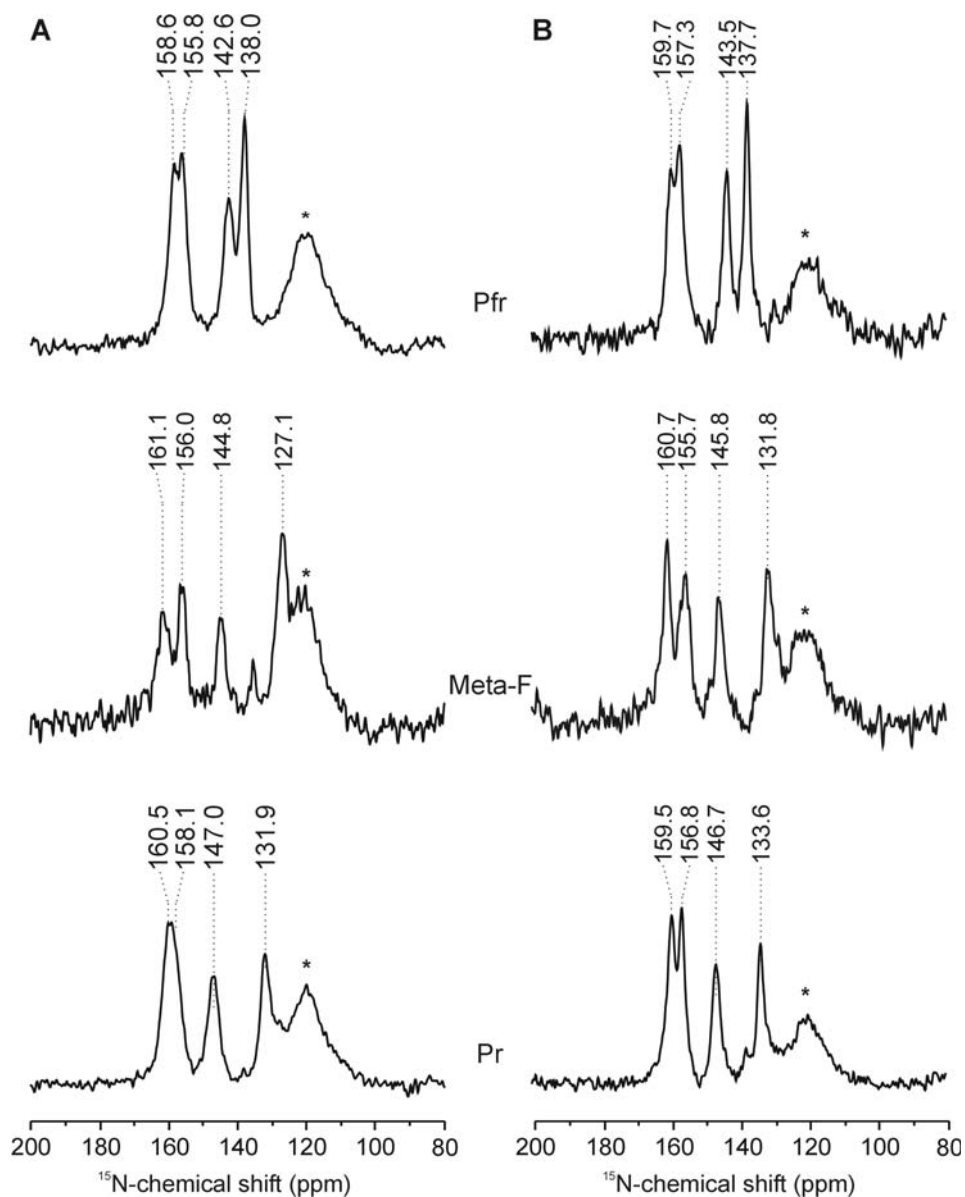


Figure 5.3: 1D ^{15}N CP/MAS NMR spectra of Cph1 Δ 2 (**A**) and *phyA65* (**B**) containing an u- ^{13}C , ^{15}N -PCB chromophore. The spectra were recorded in a field of 17.6 T and a spinning frequency of 8 kHz.

clear differences are observed at N24, especially in the Meta-F states (127.1 and 131.8 ppm for Cph1 Δ 2 and *phyA65*, respectively). In **Chapter 4**, it has been proposed that the large difference in ^{15}N chemical shift of N24 in the Meta-F and Pr states (127.1 and 131.9 ppm, respectively) is due to the weakness or even the absence of hydrogen-bonding interaction at N24 in the Meta-F state. As shown in Figure 5.3, the resonance of N24 in *phyA65* occurs at 131.8 and 133.6 ppm in Meta-F and Pr, respectively. In *phyA65*, N24 is downshifted by 1.8 ppm in the Meta-F state (compared to Pr), this downshift is significantly weaker than in Cph1 Δ 2 (4.7 ppm). Hence, it appears that in plant Phys the environment of N24 in the Meta-F state is already relatively similar to the one in Pr, while N24 is still very different in the Meta-F and Pr states in Cphs. That may explain why previous vibrational spectroscopic studies on plant Phy reported very similar spectra for Meta-F and Pr [43].

As concluding remark, the methodology described in this thesis sets the stage for studying the chromophore/protein interactions within Cphs and plant Phys during their respective photocycle. This will contribute to a better understanding of the light-induced chromophore activity and signal transduction pathway in Phys.

The mechanistic understanding of the photochemical processes in Phy may also allow for comparison with analogue studies on other photoreceptors and will allow looking into the fundamental principles of naturally occurring photo-switches. Understanding the basic principles of the natural photo-switches may also inspire construction of efficient artificial nano photo-switches.