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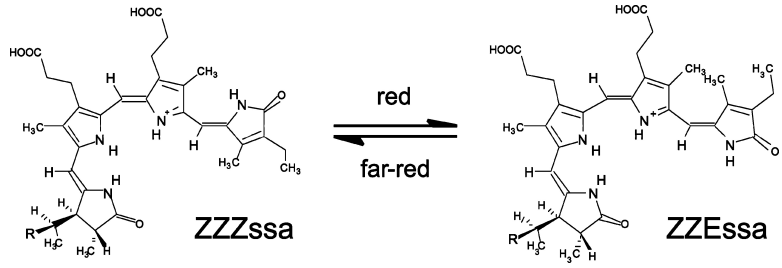

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Heteronuclear NMR Investigation on the Structure and Dynamics of the Chromophore Binding Pocket of the Cyanobacterial Phytochrome Cph1

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Abstract: Structural changes of the chromophore in phytochrome proteins associated with its photocycle are still not fully understood. We use heteronuclear NMR to investigate the conformation and dynamics of the chromophore in the binding pocket of the cyanobacterial phytochrome Cph1. On the basis of distance information obtained from three-dimensional nuclear Overhauser enhancement (3D-NOESY) spectra using the photochemically intact photosensory module of Cph1 we demonstrate that the chromophore is in the *ZZZssa* form in the P_r (red absorbing form) state and the *ZZEssa* form in the P_{fr} (far-red absorbing form) state of the protein. While *ZZZssa* for the P_r state is in agreement with a recently determined X-ray structure, no comparable information for the P_{fr} state of photochemically intact phytochrome has been available up to now. In addition, the chromophore in the binding pocket of Cph1 exhibits a notable mobility, which is distinctly different in the two photostates.

Introduction

Light is important for all living organisms, from unicellular microorganisms to higher plants and animals. On the one hand it is needed as an energy source, on the other hand it is a stimulus for a variety of vital developmental processes. Necessarily different mechanisms had to be evolved to perceive the light stimulus and to respond depending on its intensity, direction, duration, and color.[1] Several types of photoreceptors are used to accomplish these substantial tasks. They usually consist of a protein component and a prosthetic chromophoric group, typically a small organic molecule.

The first photomorphogenetic photoreceptors discovered were the phytochromes.[2,3] They regulate numerous metabolic pathways in all flowering plants by sensing red and far-red light via two spectrally different photoconvertible isoforms. A photocycle establishes a dynamic equilibrium between a red light absorbing P_r form ($\lambda_{max} \sim 660$ nm) and a far-red light absorbing P_{fr} form ($\lambda_{max} \sim 730$ nm) (where λ_{max} is the wavelength of the absorbance maximum in the red/far-red region), thereby enabling the plant to respond to a specific light quality by triggering an ATP-dependent kinase activity present in the phytochrome protein.[4,5] Although phytochromes were originally discovered in plants, it has been shown more recently that they also exist in cyanobacteria, nonphotosynthetic bacteria, and even in fungi.[6] Phytochromes are biliproteins, i.e., they contain an open-chain tetrapyrrole bilin chromophore that is covalently attached to the protein. Although the general principle of light perception appears to be the same in all phytochromes, they differ somewhat in the nature of their bilin chromophore and the way it is attached to the protein. Plant phytochromes use phytochromobilin[7] (PΦB, Figure 1a); cyanobacteria use phycocyanobilin[8,9] (PCB, Figure 1b). Both chromophores are covalently bound at C3′ to a conserved cysteine residue in the protein.[7,10–12] Nonphotosynthetic bacteria and fungi incorporate biliverdin[13] (BV, Figure 1c) via covalent linkage at C3″ to a cysteine residue upstream of the plant/cyanobacterial binding site.[14,15]

Information about chromophore−protein interactions at atomic resolution is absolutely required for a detailed understanding

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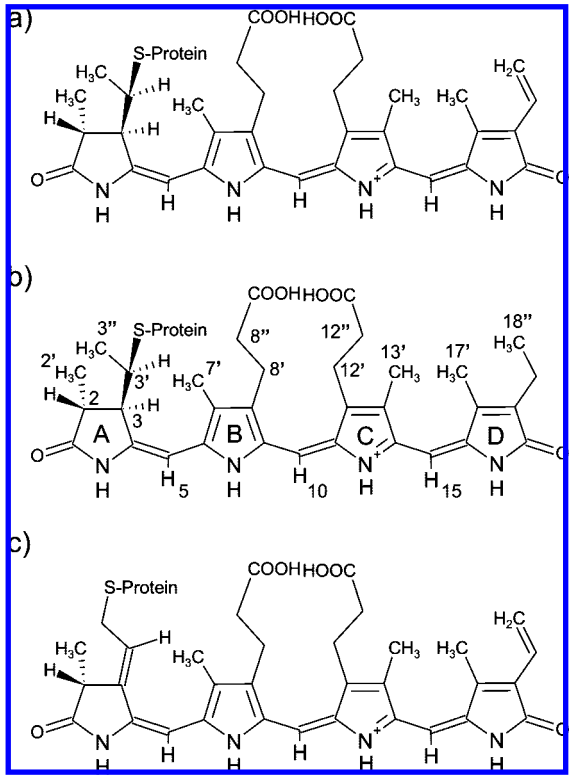

Figure 1. Chemical formulas of the three types of tetrapyrrole chromophores found in phytochrome proteins. Attachment of the chromophore to the protein takes place at a Cys residue in the protein via ring A of the chromophore. All chromophores are depicted in their protonated form. Note that only constitution, numbering, and attachment of the protein are shown, not the proper conformation, which is depicted in Figure 7. (a) PΦB is the chromophore usually found in plant phytochromes. (b) PCB is the chromophore usually found in cyanobacterial phytochromes and was used in the present study. (c) Biliverdin (BV) is the chromophore usually found in bacterial phytochromes; the attachment is depicted as found in the recent high-resolution X-ray structure of the phytochrome from *Deinococcus radiodurans* (ref 23).

of the photoconversion mechanism and its structural and functional implications concerning phytochrome signaling. Earlier vibrational spectroscopic investigations of the intact protein provided first insights into the molecular structure and led to the creation of structural models.[16–22] The crystallization of a phytochrome protein for 3D-structure determination with X-ray diffraction techniques, however, turned out to be extremely difficult. In this respect the X-ray structure of the P_r form of a bacterial phytochrome from *Deinococcus radiodurans* (DrBphP, PDB codes 1ZTU and 2O9B/2O9C),[15,23] published in 2005 and recently refined, represented a milestone in phytochrome research, although the crystallized protein did not contain the PHY domain shown to be absolutely necessary for photoconversion.[24,25] The investigated fragment was photochemically dysfunctional; thus, important protein−chromophore contacts accompanying a fully functional phytochrome protein could not be characterized.

Most recently this problem has been approached with the structure determination of the complete sensory part of the cyanobacterial phytochrome Cph1 in P_r (PDB code 2VEA, Jon Hughes, University of Giessen, Germany, and Lars-Oliver Essen, University of Marburg, Germany, personal communication). It differs from earlier X-ray structures (1ZTU, 2O9B, 2O9C) in two important aspects. First, 2VEA includes the PHY domain resulting in a phytochrome capable of photoconversion. The second difference relates to the nature of the incorporated chromophore: whereas DrBphP contains BV, Cph1 uses PCB, leading to differences regarding the chromophore attachment mode and binding site. Nevertheless, the conformation of the chromophore in both proteins turned out to be quite similar. This was predicted earlier, on the basis of the stereochemistry of the two chromophores and the high homology of the amino acids forming the binding pocket in the appropriate proteins.[26] Unfortunately, structural information for the P_{fr} form of Cph1 is presently not available.

We are using NMR spectroscopy to obtain information on the structure and dynamics of the PCB chromophore in the Cph1 binding pocket, working with the same Cph1 construct that led to 2VEA, i.e., Cph1Δ2 (the N-terminal 1−514 residue sensory module of *Synechocystis* PCC6803 phytochrome Cph1), comprising the first 514 amino acids of Cph1 and a C-terminal histidine tag. This construct is photochemically indistinguishable from the full length protein. On the basis of ^{15}N NMR spectra we could already demonstrate that the PCB chromophore is protonated in the P_r as well as the P_{fr} form of the protein. In addition, the dynamics of the chromophore with respect to an exchange of nitrogen-bound hydrogens with water, on the one hand, and to the mobility within the tetrapyrrole framework, on the other, has been investigated.[26–28] Here we present data on the conformation of the chromophore in both photoactive states. In contrast to the X-ray crystallographic investigations mentioned above, we obtained this information from studies of the functional protein in solution at room temperature. On the basis of an assignment of the resonances of the chromophore when attached to the protein we could extract distance information from three-dimensional nuclear Overhauser enhancement spectroscopy (3D-NOESY) spectra. With this we can determine the chromophore conformation with respect to the methine bridges connecting the four pyrrole rings to be *ZZZssa* in the P_r from, in agreement with 2VEA. The conformation of the P_{fr} form can be determined as *ZZEssa*, demonstrating that the only structural change that takes place in the chromophore during photoconversion is the isomerization of the double bond between the C and the D rings. Finally, the line width of the resonances in the two isoforms indicates that

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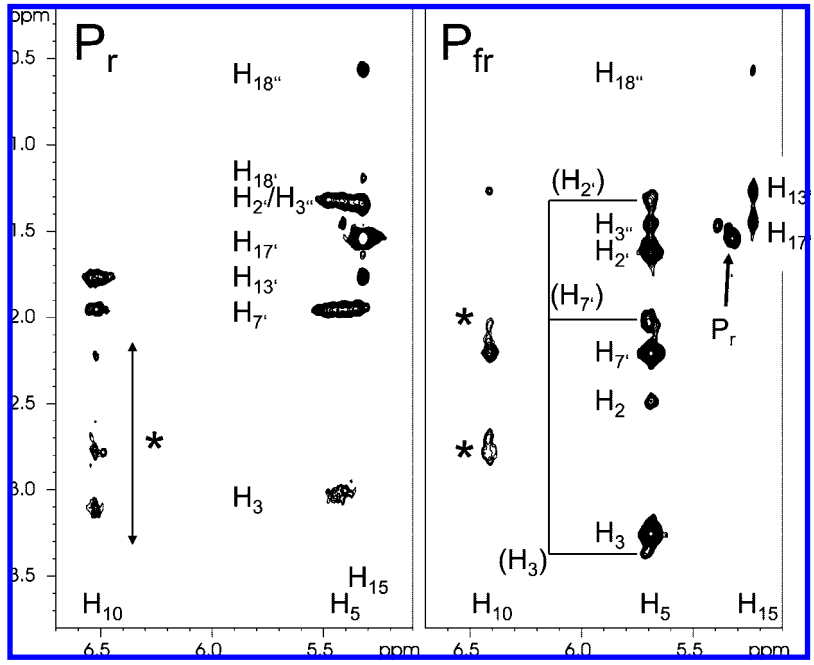

Figure 2. NOESY spectra of the P_r and the P_{fr} forms of Cph1Δ2 assembled with native PCB recorded at 900 MHz using a mixing time of 50 ms. Only correlations from the methine bridges to other hydrogens of the chromophore are shown. Assignments of the methine bridges are given at the bottom of the spectra; those of the other hydrogens are indicated in the spectra. The hydrogens of the propionate side chains are indicated by a star (∗); no attempt was made to obtain a more specific assignment.

the dynamics of the chromophore is quite unexpected and changes during the photoconversion.

Materials and Methods

The preparation of deuterated Cph1Δ2-apoprotein was based on a procedure described earlier.[11] All media components as well as antibiotics and IPTG (isopropyl-β-D-thiogalactopyranoside) were either dissolved in D_2O or, if they were only available as hydrates, lyophilized and subsequently dissolved in D_2O. An amount of 50 mL of liquid Luria−Bertani with appropriate antibiotics was inoculated with a fresh transformant of *p926.5* (Cph1Δ2 with C-terminal His_6-tag) in *E. coli* BL21(DE3)pSE111 (Novagen, EMD Chemicals, San Diego, CA; *pSE111* encodes for *lacI*^{q} and *ArgU*) and incubated at 37 °C to an OD_{600} of 1. The cells were harvested by centrifugation, washed with 150 mM NaCl, and then transferred to 500 mL of D_2O−M9-minimal medium[29] with appropriate antibiotics and perdeuterated glucose and ammonium chloride as carbon and nitrogen sources, respectively. This labeled preinduction culture is necessary for the adaptation of the cells to D_2O and for reaching a high degree of deuteration. Cells were grown at 37 °C to an OD_{600} of 1, collected again, and resuspended in 2 × 500 mL of D_2O−M9-minimal medium. After a short incubation at 18 °C protein expression was induced with 100 μM IPTG, and the cells were grown for an additional 48 h at 18 °C. Finally cells were collected again by centrifugation, quick-frozen in liquid nitrogen, and stored at −80 °C. Alternatively, expression of deuterated Cph1Δ2-apoprotein was performed with the fedbatch-pro high cell density fermentation (HCDF) system from the DASGIP AG (Jülich, Germany). This software-controlled system monitors and regulates all fermentation steps automatically, ending up in higher cell densities and protein yields. Additionally, an efficient dosage of NMR-isotope-labeled nutrients during the fermentation process is possible, leading to a significant cost reduction.[30] Preparation of native or ^{13}C,^{15}N-labeled PCB chromophore and purification of Cph1Δ2-holoprotein have been described elsewhere.[11,28]

All NMR measurements were performed using 5 mm sample tubes filled with 600 μL of Cph1Δ2 (assembled with either native or ^{13}C,^{15}N-labeled PCB) dissolved in D_2O at a concentration of about 350 μM. Prior to measurements of the P_{fr} form, the protein was photoconverted in the concentration used for NMR as described previously.[28]

Spectra were recorded either on a 900 MHz AV900 spectrometer (Bruker, Karlsruhe, Germany) using a conventional inverse triple-resonance probe equipped with a one-axis self-shielded gradient system or on a 750 MHz DMX750 spectrometer (Bruker, Karlsruhe, Germany) using a cryogenic 5 mm TXI probe equipped with a one-axis self-shielded gradient system.

NOESY spectra of the P_r and the P_{fr} forms were recorded at 900 MHz applying low-power presaturation of the residual HDO signal and using 144 scans and a 50 ms mixing time. 4096* × 256* complex data points were recorded in F2 and F1 with a spectral with of 15 150 and 12 500 Hz, respectively.

^{13}C-HMQC (heteronuclear multiple quantum correlation) spectra of the P_r and the P_{fr} forms were recorded at 750 MHz applying low-power presaturation of the residual HDO signal and using 16 scans. 512* × 64* complex data points were recorded in F2 and F1 with a spectral width 12 500 and 3570 Hz, respectively.

3D-NOESY−HMQC spectra of the P_r and the P_{fr} forms were recorded at 750 MHz applying low-power presaturation of the residual HDO signal and using a mixing time of 50 ms. For the spectrum of the P_r form, 64 scans were used and 512* × 30* × 55* complex points were recorded using 12 500, 3570, and 10 000 Hz spectral width in F3, F2, and F1, respectively. For the P_{fr} form, 80 scans were used and 512* × 25* × 57* complex points were recorded using 12 500, 3570, and 10 000 Hz spectral width in F3, F2, and F1, respectively. Carbon chemical shifts were recorded in F2 for both photostates.

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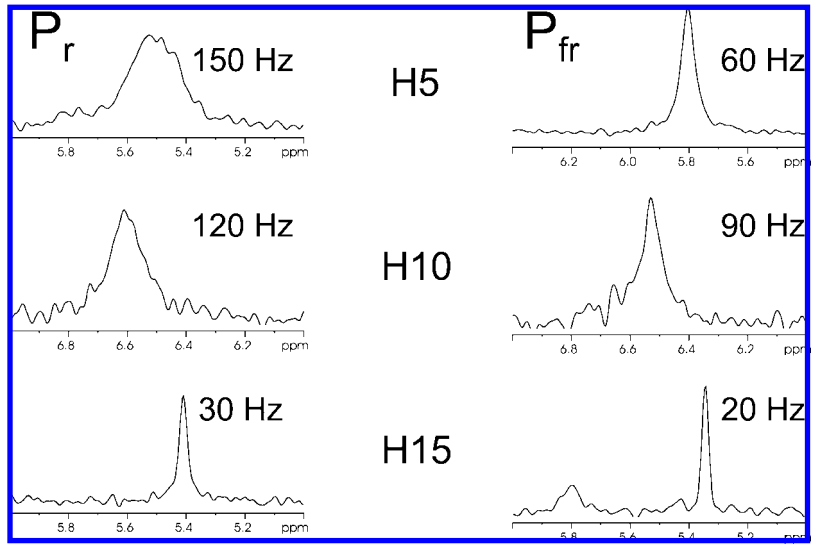

Figure 3. One-dimensional slices extracted from the NOESY spectra shown in Figure 2. The slices are taken parallel to the F2 axis and show cross sections through correlations between methine bridges and other hydrogens of the chromophore and allow for a determination of the line width of the hydrogens. In the P_{fr} form H5 at the methine bridge between rings A and B shows two lines with similarly reduced line width of which only one is shown. Semiquantitative estimates for the mobility within the chromophore are discussed in the text.

Data were processed using topspin 1.3 (Bruker, Karlsruhe, Germany) and subsequently transferred to the program Sparky[31] for analysis.

Results

Samples and NMR Spectra. For the present investigation two types of samples were utilized: In both, deuteration of the protein, Cph1Δ2, was necessary for a reliable identification of signals from the chromophore since deuteration eliminates the overwhelming number of protein hydrogen signals from the spectra. Nevertheless, a complete exchange of hydrogens by deuterium in the recombinant protein is hardly achievable. Thus, it was not possible to remove all protein signals from the NMR spectra.

In the first type of sample native PCB was assembled with deuterated Cph1Δ2 to record two-dimensional (2D), homonuclear NOESY spectra. In the second type of samples fully ^{13}C- and ^{15}N-labeled PCB was assembled with deuterated Cph1Δ2. These samples were used to record two-dimensional, heteronuclear correlation spectra (HMQC) as well as 3D-NOESY−HMQC spectra. In all cases spectra of the P_r as well as the P_{fr} form of the protein were recorded. Since the samples were prepared in the dark, the initial state of the protein was P_r that is present to 100%. Prior to measurements of the other photostate the samples had to be photoconverted. Due to overlapping absorption spectra a complete conversion from P_r to P_{fr} is not possible. Accordingly, the P_{fr} samples measured here consist of 70% P_{fr} and 30% P_r.[32] It is possible to distinguish signals from both photostates by comparing the spectra; signal overlap only occurred in a few cases. No attempt was therefore made to obtain pure P_{fr} by chromatographic separation.

As we have described earlier the quality of the spectra is negatively affected by the broad lines that the chromophore exhibits due to dynamic effects (see also below).[26] In the case of the NOESY spectra, the broad lines reduce the intensity of the chromophore peaks relative to the background signals from the protein, which are particularly strong in the region of the methyl groups. Although the spectra are still interpretable in that region, far more reliable information can be extracted from the region where NOESY correlations between the three hydrogens at the methine bridges of PCB and other chromophore hydrogens can be found (Figure 2). No protein signals appear in that frequency range.

In the 2D-NOESY spectra the remarkable dynamics of the chromophore is immediately visible from the line width of the protons H5, H10, and H15. This can be observed even better in one-dimensional traces through the two-dimensional spectra that are shown in Figure 3. In the P_r form the lines of H5 and H10 exhibit a line width of more than 100 Hz, whereas the line of H15 is only 30 Hz wide. As a direct consequence only the strongest peaks resulting from H5 and H10 are observable in the NOESY spectrum, whereas for H15 more peaks from weaker correlations can be expected. The situation changes drastically for H5 in the P_{fr} form. Whereas H10 and H15 exhibit line widths comparable to that in P_r, two lines of similar and reduced line width appear in the case of H5. This results in two sets of signals that can be distinguished in the 2D-NOESY spectra, one being substantially weaker than the other.

As can bee seen in Figure 2, signals from the P_r form are barely visible in the P_{fr} spectrum. Since H5 and H10 have very broad lines, it is not surprising that the only P_r signal visible is that of the strongest H15 peak, indicating a short distance between H15 and the methyl group in position 17′. The methyl groups have sharper and more intense lines; thus, they give rise to the majority of the signals in the 2D-NOESYs.

Broad lines also lead to reduced peak intensities in two-dimensional heteronuclear spectra. In the case of deuterated Cph1Δ2 assembled with ^{13}C,^{15}N-labeled PCB those spectra hardly contain any signals. The only heteronuclear correlation that can be recorded within a reasonable time is a ^{13}C-HMQC. It contains peaks for all methyl groups of the chromophore, which benefit from the "methyl-TROSY" effect.[33] We see 6 peaks in the P_r form and 12 in the P_{fr} form (6 from each "pure" form) as shown in Figure 4. A comparison between the two spectra shows that the chemical shifts of the carbon atoms are barely affected by photoconversion, whereas the chemical shifts of some of the hydrogens exhibit strong changes when conver-

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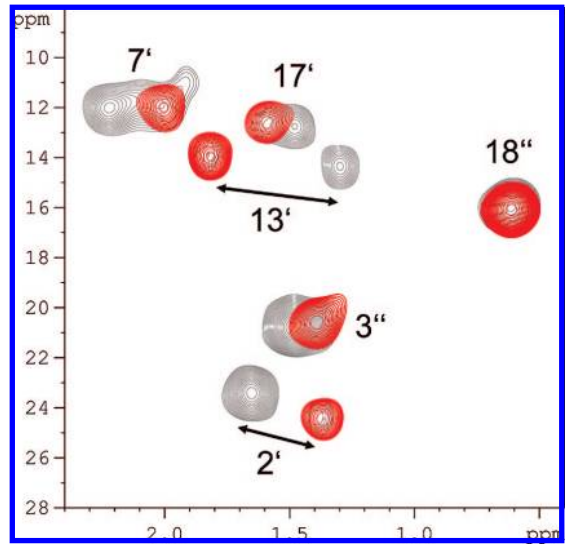

Figure 4. Overlay of the methyl region of the $^{1}H,^{13}C$-HMQC spectra of the P_r (red) and the P_{fr} forms of Cph1Δ2 assembled with $^{13}C,^{15}N$-labeled PCB. Since peaks corresponding to identical positions in PCB are close to each other assignments are given for both forms simultaneously. The increased intensity of the methyl groups 2′ and 3″ in the P_{fr} form should be noted; it indicates a reduced line width for resonances of the A ring that can also be seen in Figure 3.

sion takes place. The intensities of the peaks in the ^{13}C-HMQC made the recording of 3D-NOESY−HMQC spectra possible. The latter spectra only contain peaks between methyl groups and other hydrogens of PCB. In addition to the deuteration of the protein, the heteronuclear editing of the spectra led to a further improvement of the suppression of unwanted signals: the spectra are much "cleaner"; they solely contain signals originating from PCB. In each of the spectra six slices are relevant, taken at the positions of the methyl groups and shown in Figures 5 and 6. The spectra are shown free of background noise, but the fact that they are recorded as three-dimensional spectra results in a dramatically reduced resolution in the indirect proton dimension as compared to the 2D-NOESY spectra. A distinction between the two sets of signals of H5 in the P_{fr} form is therefore impossible in the three-dimensional spectra.

All NOESY spectra were recorded with a mixing time of 50 ms. This choice was made based on several considerations. Cph1Δ2 is large by NMR standards, and the mixing time should therefore be kept short to avoid spin diffusion. The binding pocket is deuterated, however, and PCB contains only few protons, so spin diffusion should actually not be severe. The spectra recorded with a mixing time of 50 ms show a reasonable cross peak intensity, and cross peaks resulting from two short distances were considered useful for the assignment process as well as a conservative structural interpretation (see below).

Assignment of the NMR Signals of the Chromophore. The resonance assignment is mainly based on NOESY spectra. In addition, as described above, the dynamics of the chromophore results in a different line width for different parts of the molecule and was used to distinguish between resonances. Finally, chemical shifts, particularly of the carbon resonances, could also be used. In the HMQC (Figure 4), e.g., the group of signals in the upper left area of the spectrum shows signals for the methyl groups directly attached to aromatic rings (7′, 13′, and 17′), whereas the other three signals belong to the positions 2′, 3″, and 18″. Structural information on the P_r form that was available from a homology model[26] allowed a further discrimination between different possible interpretations of the NOESY spectra. The spectra of the P_r form were assigned first and subsequently used to assign the spectra of the P_{fr} form. In the end all this information led to a consistent assignment of all peaks. This is shown in Figure 2 for all protons in the 2D-NOESY spectra and in Figure 4 for the methyl groups in the ^{13}C-HMQC. An assignment of the resonances of the propionate side chains was not possible, because they are very broad and show only interactions with the proton H10.

Distance Information from the NOESY Spectra. Due to the dynamics of the chromophore in the binding pocket it is difficult to get enough structural constraints from the NOESY data to allow for a full structure calculation. We can, however, use the distance information to determine the stereochemistry at the three methine bridges C5, C10, and C15. The established nomenclature for a description of this stereochemistry refers to the configuration of the double bond (*Z* for cis or *E* for trans) and to the conformation of the single bond (*s* for synperiplanar and *a* for antiperiplanar), and we will also use this nomenclature here (but see the discussion below).

The situation at the central methine bridge involving H10 could only be derived from the 2D-NOESY spectra (Figure 2), simply because no signals from H10 are visible in the three-dimensional spectra. The spectra do not change from P_r to P_{fr}. In both cases signals to the propionate chains at rings B and C as well as signals to the methyl groups at 7′ and 13′ are visible. The occurrence of the latter ones can be explained with the overall higher intensity of peaks in 2D-NOESY spectra as compared to the 3D-NOESYs. The appearance of H10−7′/13′ cross peaks results most likely from a two-step transfer from H10 via hydrogens at 8′ and 12′ to the two methyl groups at 7′ and 13′. According to this, H10 points toward both propionate chains, and the stereochemistry at the central methine bridge is thus *Zs* in both the P_r and the P_{fr} forms.

The interpretation of the distances at the methine bridge between rings C and D is shown in more detail in Figure 5. With two possible stereoisomers at both of the two bonds, four possible isomeric situations have to be considered. They are depicted above the spectra. Including only distances between methyl groups and methine bridges, and expecting a strong peak from a shorter distance (fat arrow) and a weak or absent peak from a longer distance (thin arrow), a unique signal pattern arises for each of the four possibilities. In the 3D-NOESY−HMQC

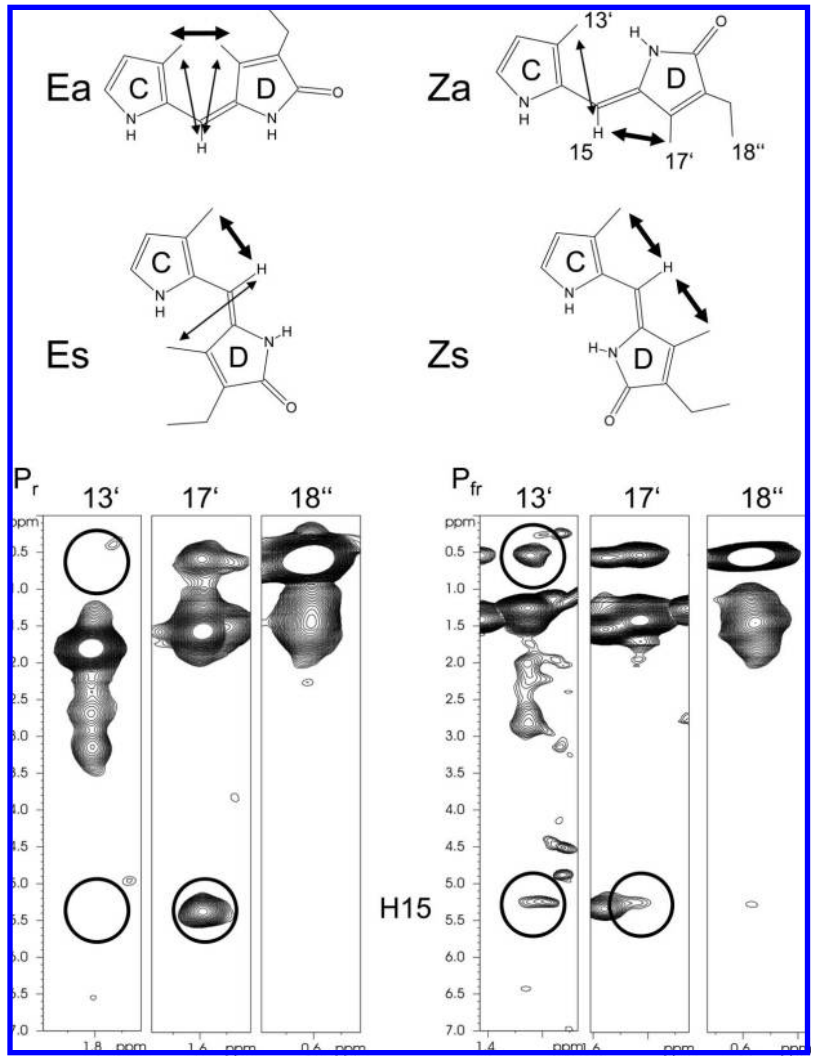

Figure 5. Interpretation of the 3D-NOESY−HMQC spectra, rings C and D. (top) Using the conventional nomenclature for the structure of tetrapyrrole moieties (see discussion) four situations can be distinguished, depending on the configuration of the double bond and the conformation of the single bond at the methine bridge. Each of the four situations translates into a different pattern in the NOESY spectra. The situation present in the molecule can then be determined by comparison with the spectra. (bottom) Slices taken from the three-dimensional spectra at the chemical shifts of the methyl groups that are given at the top of the slice. The slices correspond to 2D-NOESY spectra. The circles indicate the position of the peaks that are used for the determination of the structure. In the P_r form a strong peak is present for the interaction between H17′ and H15, whereas no peaks are present between H13′ and either H15 or H17′/H18″. This is only possible in the *Za* structure. In the P_{fr} form strong peaks are detectable for the interaction between H13′ and H17′ and H18″. Only weak peaks are present for the interaction between H15 and H13′ or H17′. Taken together, this indicates an *Ea* structure.

of P_r a strong peak is observable between the methyl group at 17′ and H15 but not between the methyl group at 13′ and H15. In the 2D-NOESY (Figure 2) a strong and a weak peak can be observed, respectively. This pattern corresponds to a *Za* stereochemistry. The situation drastically changes in the P_{fr} form. Two weak cross peaks are observable from both methyl groups. In addition, a strong NOE between the two methyl groups at 13′ and 17′ and a strong NOE from the methyl group at 13′ and at 18″ are visible, the latter resulting from a two-step transfer. The stereochemistry is therefore *Ea*.

Similar considerations can be made to derive the stereochemistry between the rings A and B as shown in Figure 6. Here the situation is somewhat different as only one methyl group (7′) is in the direct vicinity of the methine bridge. Since the hydrogen H3 and the methyl group at 2′ are fixed and close in space, a short distance between H5 and H3 will always lead to a two-step transfer from H5 to H2′. Another difference is the altered line width of H5 in the P_{fr} form that will lead to more intense peaks in the spectra. In the 2D-NOESY (Figure 2) two strong correlations are observable between H5 and H3 and H5 and H7′. In the 3D-NOESY−HMQC connectivities between H5 and H7′ can be observed; in addition, a peak between H5 and H2′ is visible that is somewhat weaker since it results from a two-step transfer. This situation corresponds to a *Zs* stereochemistry which is further supported by a stereochemical argument made previously.[26] Since the stereochemistry at C3 has been determined to be *R*,[7,34] and the attachment site of the protein Cys259 determines the approach of the side chain onto the chromophore, the binding pocket only allows either a *Ea* or *Zs* stereochemistry at the methine bridge. The former would result in a longer distance between H7′ and H5 which can be excluded from the spectra. No change is detectable after photoconversion. Since H5 exhibits a narrower line in P_{fr} the NOEs are observable more easily. As a consequence an additional NOE is observable between H5 and the 3″ methyl group that also results from a

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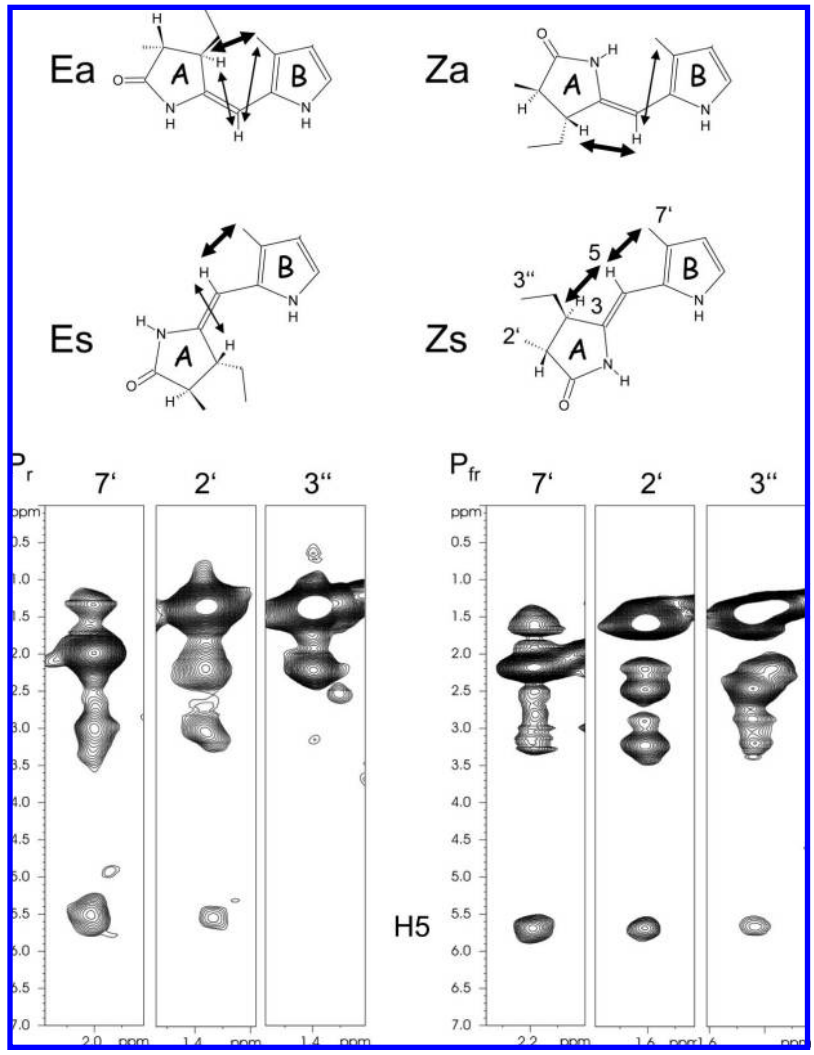

Figure 6. Interpretation of the 3D-NOESY−HMQC spectra, rings A and B. (top) The same considerations as in Figure 5 are visualized. (bottom) Slices taken from the three-dimensional spectra at the chemical shifts of the methyl groups that are given at the top of the slice. The slices correspond to 2D-NOESY spectra. In the P_r form a strong peak is present for the interaction of H7′ with H5. The peak between H2′ and H5 results from a two-step transfer and indicates a short distance between H3 and H5. This is only possible in the *Zs* structure. The pattern of peaks and their relative intensities do not change from P_r to P_{fr}, indicating that the structure is *Zs* in the P_{fr} form as well. The appearance of the peaks is different, however, since the lines of H5 are much narrower in the P_{fr} form.

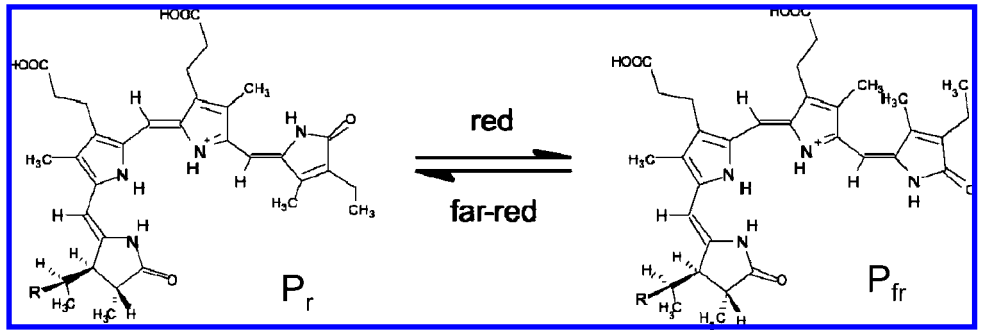

Figure 7. Structures of the two thermostable forms of phytochromes. The P_r form is given as *ZZZssa*, the P_{fr} form as *ZZEssa*. Both are names for a class of conformations as explained in the text. Both forms are shown as fully protonated chromophores as previously derived from NMR measurements (ref 26).

two-step transfer. The overall pattern of peaks, however, does not change. Similar peaks as in P_r are identifiable, and thus the stereochemistry is also *Zs*. The same can be derived from the second set of signals. Since their intensity is lower, the peaks are more difficult to observe and can only be distinguished in the 2D-NOESY.

Taking all arguments together the stereochemistry at the chromophore methine bridges can be defined and is depicted in Figure 7. In the P_r form it is *ZZZssa*. This is in agreement with the recent Cph1 X-ray structure (2VEA, Jon Hughes, personal communication) and confirms our earlier prediction that was based on stereochemical arguments.[26] The stereochem-

istry in the P_{fr} form is *ZZEssa*. This indicates that the only structural rearrangement that is taking place during photoconversion in the chromophore is a change of the configuration of the double bond between rings C and D.

Discussion

The overall conformation of tetrapyrrole chromophores such as those depicted in Figure 1 is usually described by giving the configuration of the double bonds and the conformation of the single bonds around the three methine bridges of the molecule. This description is considered sufficient since the five-membered rings are rigid units that also direct substituents in a particular direction once the orientation of the ring is defined. In addition, the aromatic system of the four rings will force the bilin to adopt an almost planar structure. In the conformation usually found for the free chromophore in organic solution, *ZZZsss*, a deviation from planarity is required due to steric effects, but otherwise the system behaves as expected.[35] If the chromophore is placed within the binding pocket of a protein obviously other effects than the aromatic system become important, mainly steric effects but also electronic effects from the amino acid side chains forming the surface of the pocket. Deviations from planarity can therefore be expected and have indeed been observed in the structures described so far. Therefore, the conventional nomenclature has to be used with care. Accordingly, defining a double bond as *Z* or *E* or a single bond as *s* or *a* results in a class of conformations with a certain range of angles instead of a single one. This is even more important since the spectra described here show that the chromophore is quite dynamic in its binding pocket, in one case (H5 in the P_{fr} form) showing two sets of signals for one of the hydrogen atoms. Even though this indicates two distinct conformations, the NOE information available shows that both are very similar and thus fall into the same class of conformations that is referred to as *ZZEssa*.

The dynamics of the chromophore does not, however, severely affect the possibility of interpreting of the NOESY spectra. Distances between the hydrogen atoms attached to the pyrrole rings do not drastically change if the relative angle between two ring planes changes by some degrees. Moreover, since we are only classifying the observable NOEs as strong or weak/absent a somewhat looser definition of the chromophore conformation as described above is possible. The results described above and shown in Figure 7 (a *ZZZssa* structure in the P_r form and a *ZZEssa* structure in the P_{fr} form) thus indicate such classes of conformations.

The structural and dynamic information on the chromophore presented in this work has been obtained for both thermally stable states of photochemically active phytochrome at room temperature. According to this, the only change of the chromophore that can be observed after the photoconversion is a flip of the D ring. In both photostates the chromophore appears to be quite mobile in its binding pocket. Although NMR spectroscopy is in principle a method well-suited for a detailed study of dynamics, this was not possible in the present study, mainly because neither the nitrogen-bound protons nor the protons at the methine bridges give rise to signals in the two-dimensional spectra usually used for an analysis of the dynamics.

Thus, only a semiquantitative interpretation is possible. Due to the covalent attachment of the chromophore the overall tumbling of the protein will dictate that of the chromophore as well. But even for a protein of that size and given that the chromophore is in a completely deuterated environment, the line width found for many of methine protons is much larger than expected. The simplest explanation is the presence of a two-site exchange with an exchange rate close to the difference in chemical shift of the two sites, i.e., a situation close to the coalescence point. As described earlier any attempts to produce sharper lines by alteration of field strength or temperature[28] have failed so far. For the H10 proton this situation does not change upon photoconversion. The sharper lines of the H15 proton in both the P_r and P_{fr} forms indicate a faster exchange as compared to H10. The situation for H5 is similar in the P_r form, but the fact that the line of the H5 proton is splitting into two lines upon photoconversion would then indicate a slower exchange between the two sites for the P_{fr} form. We note that a conformational heterogeneity is also described based on resonance Raman measurements.[37]

We cannot observe the side chains in the binding pocket and can thus only speculate what their dynamic behavior is like. It is, however, unlikely that this kind of mobility takes place in a completely rigid protein environment; thus, the whole binding pocket is most likely flexible. This is in line with recent investigation of α-phycoerythrocyanin where results from X-ray as well as NMR studies indicate a local flexibility around the chromophore and where a rigidification after photoconversion is found, corresponding to the slower exchange in Cph1Δ2.[38,39]

Given this kind of mobility it is hardly conceivable that the structural changes in the chromophore will have any substantial steric effect on the protein environment, since there is space in the binding pocket for small movements of the chromophore. The bridges between A and B rings and between B and C rings are the more rigid part of the chromophore, with an additional rigidification at the A/B bridge upon phototransformation. The bridge between the C and D rings on the other hand appears to be rather mobile, in line with the space around it in the binding pocket as found in the X-ray structures. This space is certainly necessary to allow for the rotation of ring D after excitation with red light without affecting the protein binding pocket.

The important question, then, is how the signaling process can be initiated after absorption of light, if changes in structure and dynamics of the chromophore are not likely to induce major structural changes in the protein environment. Nevertheless, structural changes have to take place to relay the light signal to the kinase domain and trigger a biological response. The most probable cause for a structural rearrangement is a rearrangement of the hydrogen-bonding network around the chromophore initiated by the flip of the D ring. While we have shown earlier that all four nitrogens in the chromophore are protonated in both the P_r and P_{fr} states, there is evidence that a deprotonation and reprotonation takes place during the photocycle.[17,36] It is well conceivable that these events accompany the rearrangement of side chains that result from altered hydrogen bonds. A clearer picture, in which the role of certain amino acids as potential partners in the hydrogen-bonding network is observable, will

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result from the soon to be available X-ray structure of Cph1 (2VEA). Changes in the hydrogen-bonding network as cause for a structural rearrangement would also imply that the changes in dynamic behavior around the A and B ring, although remarkable, are the result of a structural rearrangement rather than the cause. And although the overall photoconversion mechanism proposed here is most likely the same for all kinds of phytochromes, the dynamic behavior might be different for other phytochromes. We are currently investigating a BV-binding bacterial phytochrome to clarify this point.

Conclusion

In conclusion, we have determined the conformation of the bilin chromophore in cyanobacterial phytochrome Cph1 and have shown that the chromophore is positioned in a rather flexible binding environment. We demonstrated that the only structural change between the two thermally stable states is a rotation of the D ring. We deduced that subsequent transduction of the light signal must be due to a rearrangement of the hydrogen-bonding pattern around the D ring of the chromophore. For a more detailed description of this rearranged hydrogen-bonding pattern further structural information from both forms of photochemically intact phytochrome is required. In particular, information about the P_{fr} form and the interaction between the sensory domain and the kinase domain will be necessary to fully explain the mechanism.

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