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The Role of the Chromophore in the Biological Photoreceptor Phytochrome: An Approach Using Chemically Synthesized Tetrapyrroles

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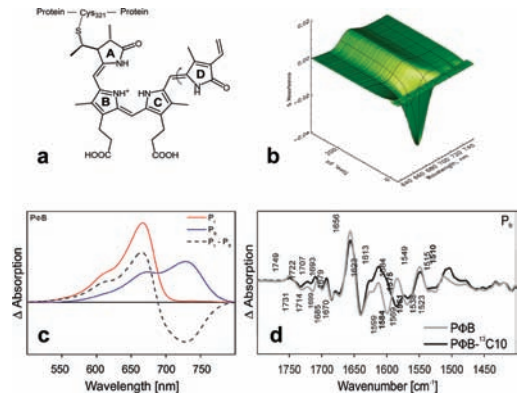
CONSPECTUS

In plants and bacteria, phytochromes serve as light-inducible, red-/far-red light sensitive photoreceptors that control a wide range of photomorphogenetic processes. Phytochromes comprise a protein moiety and a covalently bound bilin chromophore. Bilins are open-chain tetrapyrrole compounds that derive biosynthetically from ubiquitous porphyrins. The investigations of phytochromes reveal that precise interactions between the protein moiety and its bilin chromophore are essential for the proper functioning of this photoreceptor; accordingly, synthetic manipulation of the parts is an important method for studying the whole. Although variations in the protein structure are readily accomplished by routine mutagenesis protocols, the generation of structurally modified bilins is a laborious, multistep process. Recent improvement in the synthesis of open-chain tetrapyrroles now permits the generation of novel, structurally modified (and even selectively isotope-labeled) chromophores. Furthermore, by using the capability of recombinant apo-phytochrome to bind the chromophore autocatalytically, researchers can now generate novel chromoproteins with modified functions.



In the protein-bound state, the phytochrome chromophore is photoisomerized at one double bond, in the bridge between the last two of the four pyrrole rings (the C and D rings), generating the thermally stable, physiologically active P_{fr} form. This conversion − photoisomerization from the form absorbing red light (P_r) to the form absorbing far-red light (P_{fr}) − covers 12 orders of magnitude, from subpicoseconds to seconds. Such spectroscopic and kinetic studies yield a wealth of time-resolved spectral data, even more so, if proteins with changed sequence or chromophore structure are utilized. In particular, bilins with a changed substitution pattern at the photoisomerizing ring D have shed light on the chromophore−protein interactions during the photoisomerization. The mechanisms generating and stabilizing the light-induced P_{fr} form of phytochromes are now seen in greater detail. On the other hand, the use of bilins with selective incorporation of stable isotopes identify light-induced conformational motions when studied by vibrational (FTIR and Raman) and NMR spectroscopy.

In this Account, we present spectroscopic investigations that provide structural details in these biological photoreceptors with great precision and document the dynamics elicited by light excitation. This approach yields important information that complements the data deduced from crystal structure.

Introduction

Porphyrins, cyclic tetrapyrrolic structures, as found in heme proteins, are biosynthetically built from open-chain tetrapyrroles via a ring closure reaction and are degraded in the same way (Figure 1). The catabolic pathway of porphyrins starts with an enzymatic oxidative ring-opening reaction by heme oxygenases yielding biliverdin IXα (BV, **4**) and its reduction product bilirubin IXα (BR, **5**) (Figure 2). Since excretion of degraded heme compounds in animals takes place in the bile, these compounds are called bile pigments.[1] Research on bilins and their properties and metabolism started long ago, since jaundice in newborn babies and

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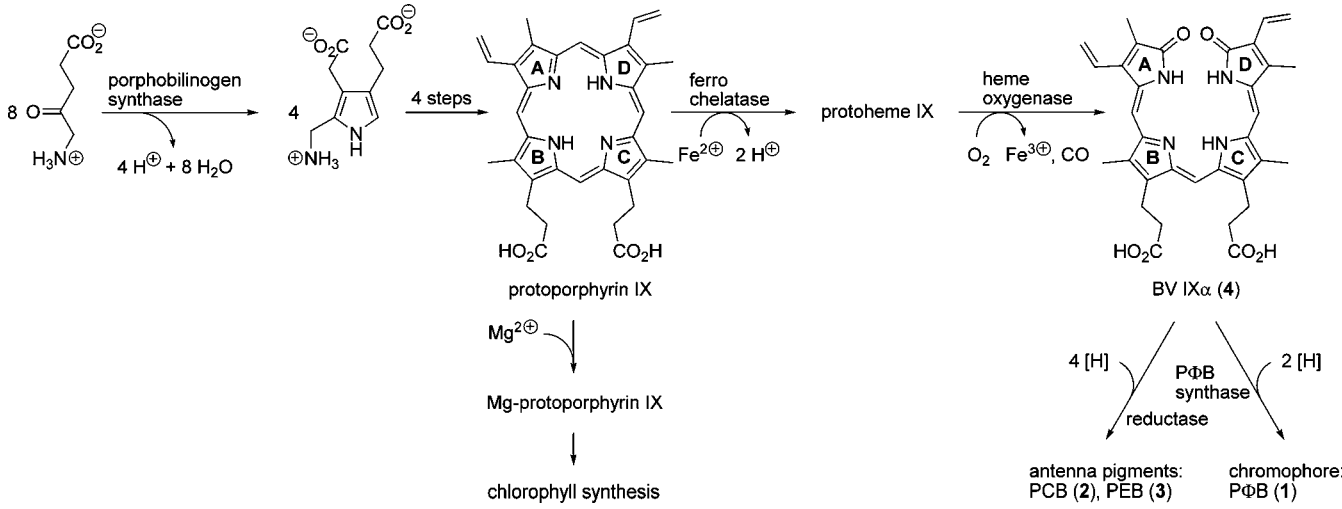


FIGURE 1. Biosynthetic pathway of the molecules essential for the generation of energy from light in photosynthetic organisms. The precursor of chlorophyll, protoporphyrin IX, is converted via two enzymatic reactions into biliverdin IXα (BV, **4**), which is reduced either to the antenna pigments **2** and **3** or to the chromophore phytochromobilin **1**.

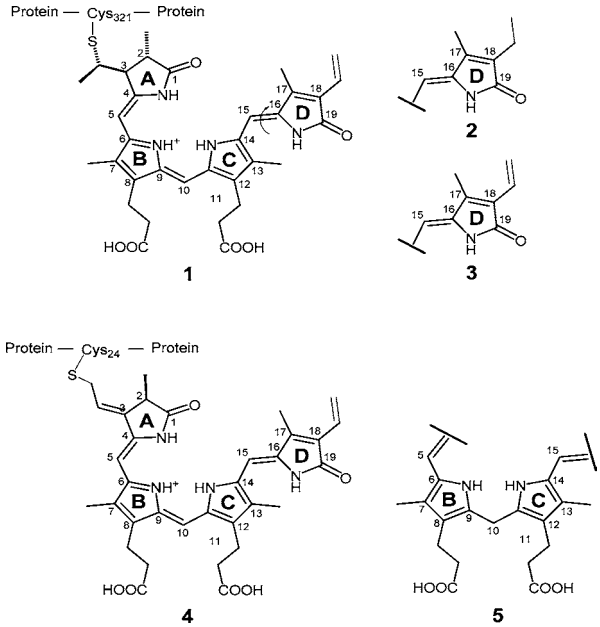


FIGURE 2. Kekulé formulas of protein-bound phytochromobilin (PΦB, **1**) and biliverdin IXα (BV, **4**) in *ZZZ,ssa* state. The photoisomerization of the C15−C16 double bond of **1** is indicated by an arrow. Additionally, the structural isomers of **1**, phycocyanobilin (PCB, **2**) and phycoerythrobilin (PEB, **3**), and of **4**, bilirubin IXα (BR, **5**), are shown. Numbering in **1** refers to oat phyA, and in **4** to phytochrome of *D. radiodurans*.

several inherited diseases can be traced to defects in this biochemical pathway.[2]

Besides appearing as intermediates in the heme degradation pathway, open-chain tetrapyrroles play important roles in biological complexes. They serve as chromophores in light-harvesting systems in cyanobacteria and are also chromophores in light-sensing photoreceptors of the phytochrome type (Figures 1 and 2). Already these two functions demonstrate the versatility of bilins, either serving as energy-transferring entities in the antenna complexes or functioning as photoisomerizing chromophores in the phytochromes. The different reactivities underscore the precise interaction between protein and chromophore; only in the protein-bound state, the phytochrome chromophore is photoisomerized within picoseconds at one selected double bond (Figure 2) to generate the thermally stable, physiologically active form.

Unfortunately, relatively few structural variations of tetrapyrroles occur naturally. The oxidative cleavage of heme yields the IXα isomer of BV (**4**), and either single (two electron)- or double (four electron)-reductions generate phytochromobilin (PΦB, **1**) or phycocyanobilin (PCB, **2**), respectively. Phycoerythrobilin (PEB, **3**), the other "mass" product in the photosynthetic antennae shows identical mass as PCB and is formed via double bond rearrangement (Figure 2).

Chromophore−Protein Interactions in Phytochromes

For nearly 40 years,[3] phytochrome studies at a molecular level were mainly directed toward plants, due to the dependence of these investigations on plant-extractable material. Since neither chromophore modifications nor its exchange is possible for holophytochrome, no structural changes could be performed. The identification of the chromophore structure was performed by NMR spectroscopy on a proteolytic fragment of native phytochrome,[4] and its biosynthesis was accomplished by inhibiting the tetrapyrrole biosynthetic pathway through the addition of gabaculine to growing plants. When such a treatment was followed by external supplementation with BV or the precursor 5-aminolevulinic acid, the native

chromophore PΦB was built and covalently attached to the apoprotein.[5]

Only with the advent of molecular biology technology, recombinant apo-phytochromes were obtained, allowing the addition of chromophores that autocatalytically attach to the correct binding site.[6−8] From a chemical point of view, the mechanism that explains best the covalent binding is a nucleophilic attack of a conserved cysteine residue to the C3′-position of the ethylidene substituent, resulting in the formation of a thio-ether (Figure 2; the figure shows also the attachment in BV-binding phytochromes, *vide infra*). The protein environment is obviously important for this reaction: in all phytochrome sequences identified so far, a histidine residue follows the covalent bond-forming cysteine, and a mutation of this residue strongly impairs chromophore attachment. A plausible proposal is that the imidazole side chain with a pK_a value in the range of the physiological pH is transiently protonated and thus provides a proton for the carbon at position 3 that gains negative charge upon the nucleophilic attack of the thiol side chain.

Molecular biology gave access to fully sequenced genomes. The first sequenced genome of a cyanobacterium, *Synechocystis* PCC6803, revealed the presence of putatively phytochrome-encoding genes in prokaryotes. Phytochrome-typical photochemistry could then be demonstrated for the recombinant, PCB-assembled gene product.[9] Following this first finding, many more phytochrome-encoding genes have been identified in the rapidly increasing number of sequenced prokaryotic genomes. Sequence analysis demonstrated the architecture of phytochromes and their physiological function as sensor proteins. With lengths of about 1100 (plant phytochromes) and between 650 and 1300 amino acids (prokaryotic phytochromes), the N-terminal half comprises the chromophore-binding domain, whereas the signaling activity was ascribed to the C-terminal part.[10] Ongoing investigations indicate the involvement also of the N-terminal region in signaling, at least for plant-derived phytochromes.[11] The N-terminal half of phytochromes contains three important protein domains: a PAS or PAS-like domain at the very front end, a GAF domain, and a phytochrome-specific PHY domain [the abbreviations PAS and GAF indicate tertiary structure motifs in proteins; they have been named after their first identification in PerArntSim-proteins (PAS) and in cGMP-specific cyclic nucleotide phosphodiesterases, adenylyl cyclases, and FhlA proteins (GAF)].[12,13] The GAF domain contains the canonical sequence of phytochromes for chromophore binding.

The majority of the prokaryotic phytochromes studied so far carry a histidine kinase activity as the signaling domain. Histidine kinases, together with response regulator proteins of the CheY-type, represent the prominent "two-component system" in many prokaryotic species.[14] The initially identified prokaryotic phytochromes carried a binding site in the GAF domain. Later on, proteins with phytochrome-type architecture and photochemistry were found that bind the chromophore, in this case BV, via a cysteine located in the PAS domain.[15]

Although PΦB is the naturally occurring chromophore in the phytochromes of all higher and probably most lower plants, its 18′,18″-dihydro derivative PCB was immediately used for assembly reactions with recombinant apophytochromes. The shorter π-electron system of PCB yields phytochromes with ca. 10−15 nm hypsochromically shifted absorption maxima for P_r (red light-absorbing form) and P_{fr}, (far-red light-absorbing form), with otherwise similar spectroscopic and kinetic properties. Interestingly, PCB, instead of PΦB, has later been found as the native chromophore in two algae, *Mesotaenium caldariorum*[16] and *Mougeotia scalaris*.[17] Another reason for applying PCB in assembly experiments is the difficult generation of the native chromophore PΦB. Besides the *de-novo* chemical synthesis (remarkably laborious and thus disqualified as a routine procedure), PEB, one of the two light-harvesting antenna constituents, is the only naturally occurring compound from which PΦB could be prepared. When treated with mercury salts, PEB forms, during an ill-defined reaction, moderate amounts of PΦB.

"Classical" phytochromes are biosynthesized as their P_r form (λ_{max} 665 nm; all data for plant phytochromes refer to the phytochrome synthesized in etiolated plants, phyA, in particular that from oat, *Avena sativa*). Irradiation of the P_r form yields, with a quantum yield of ca. 17%, the physiologically active P_{fr} form (λ_{max} 728 nm). This conversion, including the picosecond photoisomerization of the C15−C16 double bond of the chromophore and a series of thermally formed intermediates, requires several hundreds of milliseconds for the full development of P_{fr}.[18,19] P_{fr} is also photochemically active and can be reconverted into the P_r parent state. Both stable species, P_r and P_{fr} from phyA, as well as the transient intermediates were studied in great detail by a variety of spectroscopic methods, such as optical, Raman, FTIR, and NMR spectroscopies.[20−24]

The Total Synthesis of Bilin Compounds

As outlined above, the complex substitution patterns of BV, PCB, and PΦB make the total syntheses of modified chromophores a challenging task. Pioneering work in the total synthesis of open-chain tetrapyrroles was performed by the

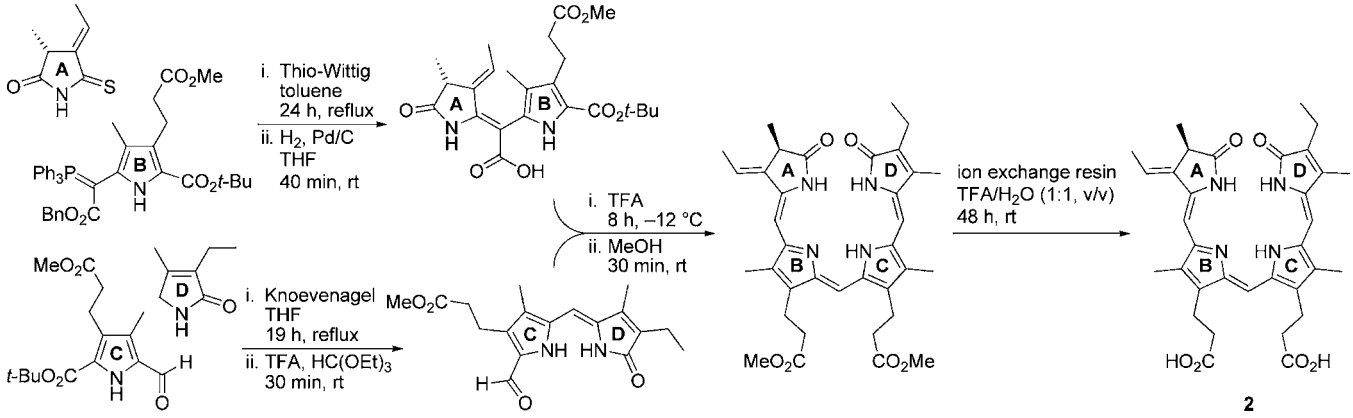


FIGURE 3. Advanced synthesis of open-chain chromophores based on the convergent strategy established by Gossauer et al.[26]

groups of Falk[25] and Gossauer,[26] who generated full-length bilins by multistep reaction pathways. The "convergent synthesis", initially presented by Gossauer, allows maximum variation of the bilins (Figure 3). It is based on the generation of each of the four pyrrole rings separately.[27,28] Rings A and B are condensed to yield the "left half" of the bilin,[27,29] and rings C and D are connected to generate the "right half". Finally, both dipyrrole units are linked via formation of the central methine bridge to afford a full-length tetrapyrrole.[30] This route allows addressing virtually each position of a bilin, an important aspect also for the selective introduction of stable isotopes.

Occasionally, a cleavage reaction of BV or PCB can be advantageous: treatment with thiobarbituric acid splits tetrapyrroles at their central methine bridge, yielding both halves of the bilin separately (Figure 4).[31] This reaction has been used to generate "iso"-PΦB (**9**) and -PCB (**10**) (see below). Another obstacle in the original synthetic approaches had to be overcome for the application of these compounds as phytochrome chromophores. The total synthesis by Gossauer et al. yielded the propionate dimethyl esters as final products.[26,27] Assembly studies with recombinant apophytochrome, however, clearly demonstrated the absolute requirement of the free propionate side chains at rings B and C.[32] This fact was confirmed by the three-dimensional crystal structure of a bacterial phytochrome revealing strong hydrogen bonding and electrostatic interactions between the propionate groups and the protein (Figure 5).[33] The ester hydrolysis was difficult to accomplish. Only the treatment with an acidic ion-exchange resin led to hydrolysis in relatively good yield.[34]

Several improvements of this fairly laborious synthetic route have been presented, preferentially aiming at reducing the number of starting materials. In recent years, important contributions have been made by the groups of Inomata and Jacobi, who published detailed overviews of their work.[35,36]

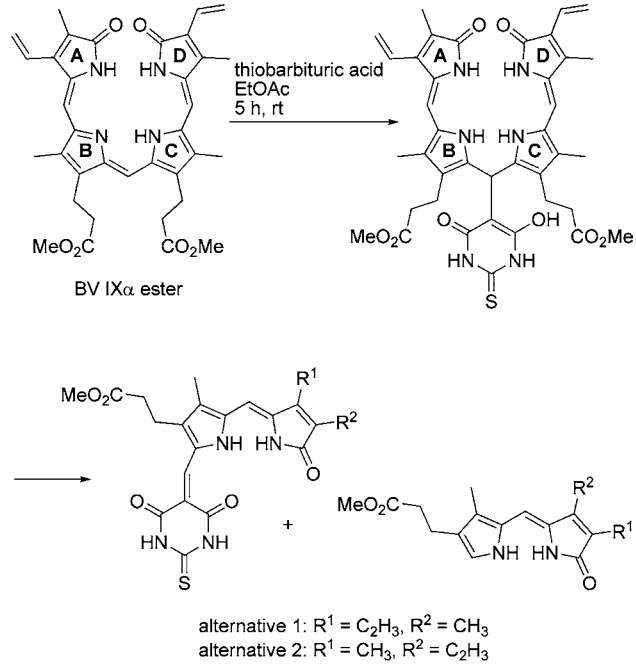


FIGURE 4. Cleavage of BV IXα ester by reaction with thiobarbituric acid.

Quite innovative synthetic approaches for strongly modified tetrapyrroles were introduced by Inomata (see below), also aimed at reducing the number of building blocks,[35,37] and Dawadi and Lugtenburg introduced an extended Knoevenagel- and a Wittig-type reaction for the generation of pyrroles.[38,39]

Building an open-chain tetrapyrrole by convergent synthesis requires all pyrrole compounds with their correct substitution pattern, as well as activation of the pyrroles at selected positions. Whereas rings B, C, and D are synthesized relatively readily and are also fairly inert during the reaction pathway, ring A demands special care, in particular considering the ethylidene substituent at position C3, essential for covalent attachment to the protein. Thus, reaction conditions should be avoided that favor a rearrangement forming 4-ethyl-3-methyl-

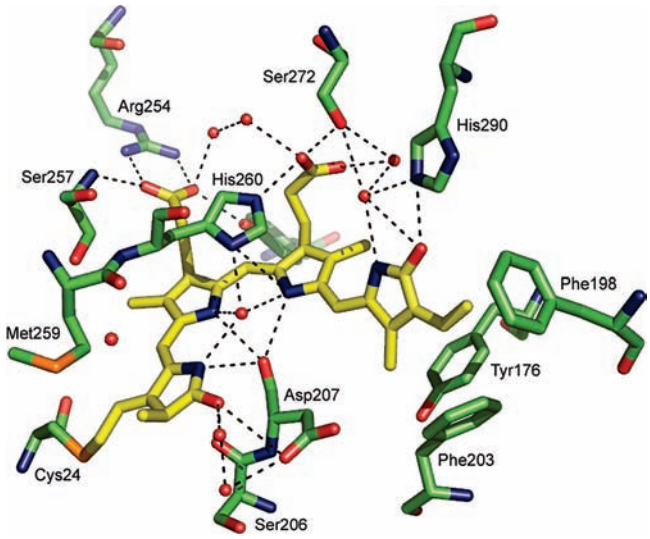


FIGURE 5. Three-dimensional structure of the PAS-GAF domains of phytochrome from *Deinococcus radiodurans* in its P_r state (PDB reference number 2o9c), showing the dense network of hydrogen bonds and electrostatic interactions between the BV chromophore (**4**, in yellow) and the protein. Adapted from ref 60.

2*H*-pyrrol-2-one. In addition, since ring A is either synthesized via cyclization of 3-cyano-2-methyl-3-pentenoic acid amide[27] or from citraconic anhydride,[40] an α,α′-carbonyl-imine or an α,α′-dicarbonyl compound results. For either compound, the connection with the ring B building block should be directed such that double bond formation takes place at the α-position next to the ethylidene substituent. A discrimination between both carbonyl groups (citraconic anhydride route) or between the carbonyl and the imino group (pentene amide route) is essential. Since the condensation between rings A and B proceeds via a Wittig-type reaction, a greater reactivity can be introduced by converting the carbonyl group or the imine function adjacent to the ethylidene into a thiocarbonyl group. The carbonyl−thiocarbonyl compound obtained thereby reacts with a triphenylphosphanylidene-1*H*-pyrrole in a Wittig reaction, yielding a 5-benzoic ester-9-*t*-butyl ester-protected AB dipyrrole. The generation of the triphenylphosphonium substituent has recently been improved by using benzyl bromoacetate,[41] which, after conversion into the iodo derivative, reacts with the ring B precursor in a radical reaction yielding the benzyloxycarbonylmethyl substituent (Figure 6). Subsequent chlorination and conversion into the triphenylphosphonium compound completes the preparation of this compound for the rings AB condensation.

Condensation of rings C and D is easily accomplished by a Knoevenagel reaction between the ring D synthon 3,4-dialkyl-1,5-dihydro-2*H*-pyrrol-2-one and a ring C compound formylated at position C5. After that, formylation of the "right half" (trimethyl-*o*-formate in TFA) and condensation with the "left half" yields the target tetrapyrrole. The removal of the propionate methyl esters completes the total synthesis (Figure 3).

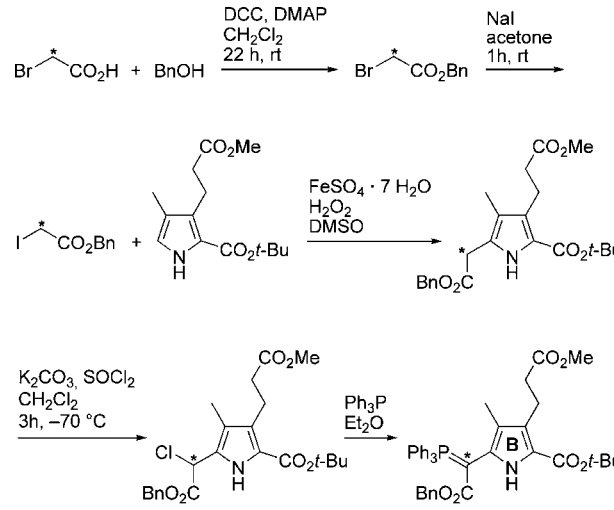


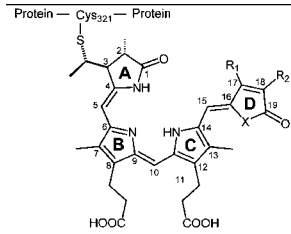
FIGURE 6. Synthesis of ^{13}C-labeled ring B.[41] The labeled carbon (∗) will be located at position C5 of the chromophore.

Effects of Chromophore Structural Changes on the Function of Phytochrome

The above-mentioned use of PCB instead of PΦB for the assembly reaction revealed indeed a significant effect of this minute structural variation, ethyl vs vinyl. Besides the simple hypsochromic shift of ca. 15 nm found for both forms P_r and P_{fr}, a change in the kinetic behavior of the PΦB- and PCB-assembled phytochromes was detected. In native and in PΦB-assembled phytochrome, the first product of the photoisomerization, lumi-R or I_{700}, decays in a biphasic manner with ca. 10 and 100 μs lifetimes (10 °C). With PCB, this process becomes monoexponential, lacking the 10 μs kinetics seen for PΦB.[42] Probably, PΦB experiences an electronic interaction between its 18-vinyl group and the protein, which, after photoisomerization, needs to be rearranged (10 μs component), whereas for PCB such an interaction does not exist. The different interactions between PCB chromophore and protein are more remarkable in the femtosecond-to-picosecond time range, probing directly the photoisomerization.[19] Bleaching of P_r is detected, but the oscillator strength of the I_{700} intermediate absorption is strongly reduced with respect to that of I_{700} in native phytochrome and is practically absent for the "iso"-PCB-carrying (**10**) phytochrome.[19] This finding immediately demonstrates the need for systematic changes of the chromophore structure (Table 1).

A major target for structural changes is definitively ring D in PΦB. The simplest change was achieved by condensing the "left half" of BV with a synthetically generated AB dipyrrole. As

TABLE 1. P_r and P_{fr} Absorption Maxima of Apo-phyA from *Avena sativa* Assembled with Chromophores Having Different Substitution Patterns



Chromophore	X	R^1	R^2	λ_{max} Pr [nm]	λ_{max} Pfr [nm]
1 (PΦB)	NH	CH_3	C_2H_3	665	728
2 (PCB)	NH	CH_3	C_2H_5	653	717
6	NH	CH_3	CH_3	655	714
7	NH	CH_3	$CH(CH_3)_2$	650	718
8	NH	CH_3	$C(CH_3)_3$	651	719
9 (iso-PΦB)	NH	C_2H_3	CH_3	663	714
10 (iso-PCB)	NH	C_2H_5	CH_3	658	707
11	NH	$CH(CH_3)_2$	CH_3	550	705
12	O	CH_3	CH_3	635	670
13	O	CH_3	C_2H_5	634	669
14	S	CH_3	CH_3	633	690
15	S	CH_3	C_2H_5	629	690
16	CH_2	CH_3	CH_3	626	641
17	CH_2	CH_3	C_2H_5	629	637

outlined above, such building blocks are obtained via thiobarbiturate cleavage of bilins (Figure 4). The product was called "iso"-PΦB (**9**), due to its reversed substitution pattern at ring D: 17-vinyl-18-methyl.[43] This compound formed a photoactive phytochrome upon assembly with the apoprotein but, selectively the P_{fr} form showed a hypsochromic absorption shift (λ_{max} 714 nm vs 728 nm in native P_{fr}), whereas the P_r absorption remained nearly unchanged (λ_{max} 663 nm). The same phenomenon was observed for the corresponding "iso"-PCB (**10**) exhibiting a nearly undisturbed P_r absorbance and a slight hypsochromic shift of the P_{fr} form (λ_{max} 707 nm vs 715 nm for PCB). Extension of this approach led to a systematic variation of substituent sizes at ring D. Changes introduced at position C18, reducing the size by attaching a methyl group, but also by larger substituents, for example, *i*-propyl or *t*-butyl, are accepted in the protein-binding site with only moderate changes in the absorption maxima of either P_r or P_{fr}. When, however, the substituent at position C17 was increased in size to the *i*-propyl group (**11**), a strong hypsochromic shift of ca. 100 nm for the P_r form was found (λ_{max} 550 nm), whereas P_{fr} showed an only moderate change (λ_{max} 705 nm). Although entirely unexpected, it concurs with formerly reported data on the assembly of phytochromes, when PEB was used as chromophore.[44] This bilin, carrying a saturated bridge between rings C and D (**3**, Figure 2), cannot undergo a photoreaction but forms a highly fluorescent P_r species absorbing at 576 nm. The strong hypsochromic shift of the absorption maximum is apparently due to the shorter conjugated π-system, extending only over the three pyrrole rings A, B, and C. We thus concluded that the substituent in 17-*i*-propyl PCB (**11**) induces a strong steric hindrance upon incorporation into the protein, forcing ring D into a conformation impairing full electronic conjugation with the rest of the chromophore. In contrast to PEB, 17-*i*-propyl PCB (**11**) can photoisomerize and then occupies a region in the protein that tolerates the larger substituent.

Similar investigations have been reported by Inomata et al.,[35,45] who presented an even wider range of modifications also at rings A, B, and C. A change of the ethyl group at position 18 (*n*-propyl or *n*-pentyl) did not alter the spectral properties of the assembled holoproteins. Only an *n*-octyl substituent caused a reduction of the P_{fr} absorption intensity.

Yet, this chromoprotein still showed photochemical activity. Similar to our studies, an increase in size of the substituent at position C17 by introducing an *n*-propyl, *n*-pentyl, and *n*-octyl group causes significant changes: whereas the *n*-propyl group is still tolerated, an *n*-pentyl group causes loss in intensity of the P_{fr} form with the P_r still bleached. No chromoprotein formation was observed for an *n*-octyl substitutent. The authors also proved the importance of the presence of the propionate side chains in unesterified form.[45] The above-discussed small structural change between PCB and PΦB was again found important for plant physiology.[35,46] Chromophore-deficient *Arabidopsis* plants could only partially be rescued in their phyA activity when PCB was added. Full activity could only be restored upon addition of PΦB, whereas the physiological function of phyB was fully established with either of the two chromophores.

Combining Chemical Synthesis with Three-Dimensional Structure Information

Screening sequenced microbe genomes for putatively phytochrome-encoding genes has revealed phytochromes in many microorganisms and has also yielded phytochromes with an entirely modified binding concept. These novel phytochromes bind covalently BV as chromophore via a cysteine in the N-terminal PAS domain.[33,47] Homologous expression of a cyanobacterial phytochrome, CphB from *Tolipothrix* PCC7601, clearly revealed that BV is the genuine chromophore in this phytochrome class.[48] The photochemical function of these BV-binding pigments was initially demonstrated for the gene products from *Deinococcus radiodurans*, *Tolypothrix* PCC7601, and *Agrobacterium tumefaciens*.[15,47,49] All these BV-binding phytochromes lack the conserved cysteine residue in the GAF domain. However, a single point mutation introducing a cysteine at the correct position in the GAF domain renders these proteins into typical phytochromes; that is, they now bind PCB covalently and show the phytochrome-typical photochemistry.[49] Irrespective of the different chromophore employed and the different binding position, most of these BV-containing prokaryotic phytochromes show both parts of the bacterial two-component system, that is, a histidine kinase and a response regulator, and were proven to undergo auto- and transphosphorylation.[48]

These BV-binding proteins proved to be structurally very robust. In fact it was a construct consisting of the PAS and GAF domains from the *Deinococcus radiodurans* phytochrome that could be crystallized as the first part of a phytochrome ever (Figure 5).[33] The three-dimensional structure clearly revealed the covalent attachment of BV at its C3″-position, an extended, still slightly bent chromophore conformation, and strong interactions between the propionate side chains and amino acid side chains. The nitrogen atoms of pyrrole rings A, B, and C are all hydrogen bound and immobilized, leaving only ring D loosely stabilized and ready for photoisomerization. The authors of this crystallization study also offered a plausible explanation for the use of BV or PCB as chromophores in the two phytochrome classes. When a cysteine residue was modeled into the GAF domain at the generic chromophore-binding position (occupied by Met_{259}), the thiol group came in close contact to the C3′-position of the bilin, making covalent bond formation highly probable.[33]

An inspection of the binding site of the crystallized *Deinococcus radiodurans* phytochrome domain reveals strong interactions between rings A, B, and C but does not yield a mechanism to explain how the photoisomerizing ring D might be fixed in its conformation in P_r and in P_{fr}. In fact, it turned out that the PAS-GAF portion of a BV-binding phytochrome shows a modified photochemistry. Upon irradiation with the appropriate wavelength, the P_r absorbance was reduced, but an only very weak absorbance increase for the P_{fr} form was observed, whereas a "classical" PCB-binding construct of the same size (i.e., comprising the PAS and GAF domains) showed P_r and P_{fr} absorbances of similar intensity.[50] Thus, it seems that for these truncated proteins, either the P_{fr} form of BV-binding phytochromes has strongly different absorption properties or the photoconversion is not complete and only yields an intermediate with a reduced oscillator strength of the P_{fr} band.

Quite a number of substituent-modified bilins were synthesized and were shown to form photoactive chromoproteins, in some cases, however, with significantly changed P_{fr} forms (Table 1). We thus suggested that the nitrogen atom in ring D with its attached proton might contribute stability to P_{fr} via hydrogen bond formations. Accordingly, a series of PCB derivatives was synthesized, carrying at ring D either an oxygen (**12**, **13**), a sulfur atom (**14**, **15**), or a CH_2 group (**16**, **17**) instead of the nitrogen atom. Each of these PCB derivatives would show reduced polarity in the D ring, and none of them would be able to establish hydrogen bonds with the protein. The synthesis yielded the designed D-ring-thia-, oxa-, and carba-derivatives of PCB,[51,52] which all bound covalently to the apoprotein with P_r forms absorbing between 626 and 635 nm, as was proven by adding PCB to the assembled chromoproteins. The effect of introducing heteroatoms in ring D became clearly visible upon irradiation. Following the reduction in polarizibility ($S > O > CH_2$), these pigments showed reduced photochemical activity. Whereas for the thia deriva-

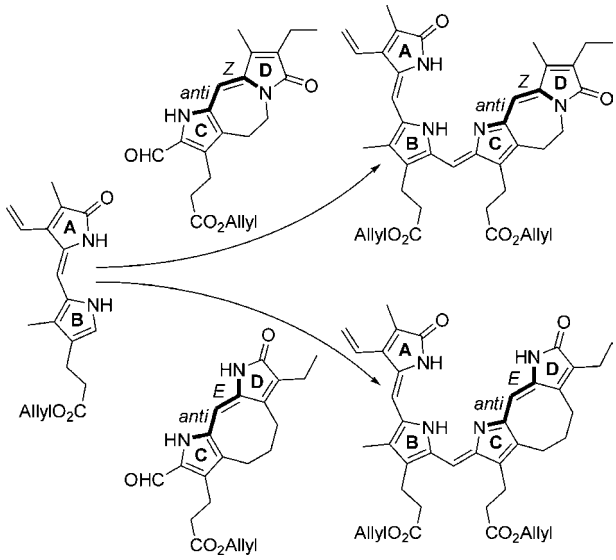


FIGURE 7. Synthesis of BV esters in sterically fixed form, obtained by connecting the rings C and D. The methine bridge between ring C and D in the upper chromophore is locked into *Z-anti* configuration and that in the lower chromophore into *E-anti*.[61,62]

tive a clearly distinguishable and for the oxa derivative a still detectable P_{fr} form could be generated, practically no photochemical activity was observed for the carba derivative containing phytochrome (Table 1). Apparently, the nitrogen atom in the D ring with its attached hydrogen atom serves as proton donor in a hydrogen bonding network that adds significant stability to the P_{fr} form of phytochromes. In fact, since the crystal structure does not reveal hydrogen atoms and their interactions, this chemical approach is a very strong argument for the role of hydrogen bonds in stabilizing the P_{fr} form.

A more detailed view on the chromophore−protein interactions has been obtained from the crystal structure of a larger phytochrome fragment, that is, the PAS-GAF-PHY portion of the cyanobacterial Cph1.[53] This construct undergoes phytochrome-specific photochemistry identical to the full-length protein, and the crystal structure revealed additional interactions between chromophore and protein that documented the stabilization of the chromophore in the P_{fr} state.

An alternative approach to understanding the chromophore conformation and the spectral properties of phytochrome was the synthesis of structurally fixed BV derivatives of various conformational orientations by covalently connecting two adjacent rings of the chromophore.[54] The additional ring, e.g., between rings C and D (Figure 7) allows fixing the chromophore in its double and single bond geometries. As a consequence, it should generate light-stable phytochromes. When the 15-*Za*-, 15-*Ea*-, 15-*Zs*-, or 15-*Es*-stabilized BV derivatives were used for chromoprotein assembly, the conformation and the configuration of the chromophore in the P_r or P_{fr} state

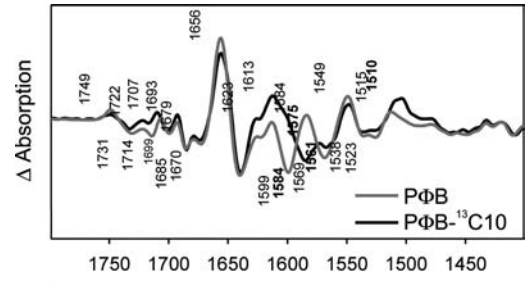


FIGURE 8. FTIR difference spectrum ($P_{fr} - P_r$) of C10-^{13}C-labeled PΦB assembled with apo-phyA of *Avena sativa* (modified from ref 55). Positive bands are assigned to the photoproduct (P_{fr}), whereas the negative bands are related to the P_r state.

could clearly be demonstrated: assembly of the apoprotein with the 15-*Za* chromophore yielded an absorption band reminiscent of the P_r form (λ_{max} 714 nm) but exhibiting a narrower bandwidth. Accordingly, the 15-*Ea* derivative generated the P_{fr} form (λ_{max} 739 nm), but interestingly, none of the 15-*syn* derivatives led to the formation of chromoproteins.[54]

Isotope-Labeled Bilins for Studies of the Spectral Properties of Phytochromes

Compounds carrying ^{13}C or ^{15}N isotopes or both at selected positions or showing a uniform labeling are valuable tools in spectroscopic investigations and allow one to monitor also the conformational changes upon irradiation. Introduction of isotopes at selected positions of a bilin again refers to the convergent route of total synthesis (Figures 3 and 6). Whereas chemical synthesis could provide access to selected chromophore positions, fully ^{15}N- or ^{13}C-labeled chromophores are obtained when appropriate isotopes are added to the growth medium of photosynthetic organisms that employ PCB as a mass pigment in their antennae.

The synthetic approach, in principle, allows the detection of a single atom in a protein with ca. 10000 atoms due to its isotope shift in bands obtained from vibrational spectroscopies, as shown by the introduction of a ^{13}C isotope at position 10 (central methine bridge, Figure 8). The FTIR analysis clearly shows the changes of PCB during the P_r-to-P_{fr} conversion[55] but also demonstrates the difficulty encountered for spectral band assignment. The introduction of a single ^{13}C isotope causes multiple band shifts, indicative of the high degree of vibrational coupling in these compounds.

When, on the other hand, a uniformally ^{13}C- and ^{15}N-labeled chromophore is incorporated into apophytochrome, the chromophore conformation and its dynamics can be probed by solid-state NMR spectroscopy, whereas the entire protein moiety remains "in the dark".[22] When exclusively ^{15}N isotopes were introduced, all four nitrogen atoms of the pyrrole rings of PCB

became observable by ^{15}N NMR spectroscopy.[22] All four NMR signals were found close together in the same ppm range of the NMR spectrum for the P_r and also for the P_{fr} state [P_r, 158.5 ppm (double intensity), 146.8 ppm, 132.1 ppm; P_{fr} 156.9 (double intensity), 142.8 ppm, 137.8 ppm] indicating that all pyrrole rings are protonated in both phytochrome states. The coalescence of two signals in both phytochrome states points to a practically identical electronic structure for two rings. We initially assigned these signals to rings B and C, which implies that the chromophore responds to the photoisomerization of the C15−C16 double bond by a conformational change around the rings A and B as compensation of the sterical constraints. A selective labeling at the C5 position, that is, the methine bridge between rings A and B and of the nitrogen atom in ring A, however, revealed that the A- and B-rings do not change in their electronic configuration, in contrast to the initial suggestion.[56] From a uniformly labeled PCB chromophore, chemical shift changes were obtained for each position of the chromophore. Such approach reveals that the photochemically induced conformational changes of ring D are compensated by torsional motions of ring C, leaving the "left side" of the bilin solidly fixed within the binding pocket.[56] The same isotope labeling (^{13}C at C5) contributed to the geometry assignment of the single and double bonds between rings A and B. This assignment requires an interpretation of the position of the C═C stretching bands employing Raman spectroscopy. The particular position of one double bond depends on its stereochemistry and that of the neighboring single bond. Initial band assignment making use of *ab initio* calculations for the chromophore in vacuo led to the erroneous assignment of this double bond as *Z* and the adjacent single bond (C5−C6) as anti (*ZZZ,asa* in ref 57). Only the use of QMMM calculations, including the entire protein, and the comparison of the spectra of the unlabeled with a $^{13}C_5$-labeled PCB chromophore allowed disentangling the C═C double bond region. The theoretical calculation of the experimentally obtained band positions point to a strong distortion of the methine bridge between rings A and B, still adopting a *syn* geometry.[58] Similarly as discussed above for the movement of the pyrrole rings during the photoconversion, we could show by FTIR and NMR spectroscopy that in both stable states, P_r and P_{fr}, the carbonyl groups firmly remain as C═O double bonds.[56,59] This is valuable information for the still open question regarding which factors determine the strong bathochromic shift upon P_{fr} formation. We cannot provide a clear-cut explanation for this observation, however, our data allow to rule out any tautomeric forms including positive charge localization at ring D, as was proposed to extend the double bond conjugation, attempting to explain the additional red shift of the P_{fr} absorption.[13]

Conclusion

Research on phytochromes has made significant progress in recent years through the application of molecular biology technology, the knowledge of the three-dimensional structure of the chromophore-binding fragment, and the application of sophisticated spectroscopic methods, all in conjunction with quantum chemical calculations. Supplementing these approaches with structurally modified or isotope-labeled chromophores now allows expanding our view also regarding the dynamics of these important photoreceptors upon light excitation, demonstrating not only the beauty of their structure but also the precision by which their conformational changes are controlled.

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BIOGRAPHICAL INFORMATION

Christian Bongards was born in 1973. After having completed a training as chemistry laboratory assistant, he studied chemistry at the University Duisburg-Essen. In 2007, he obtained his Ph.D. in chemistry from the University of Düsseldorf for his experimental work carried out at the Max-Planck-Institut für Bioanorganische Chemie in Mülheim, where he worked as a postdoctoral fellow until 2008. He is now at Sanofi-Aventis.

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FOOTNOTES

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