Agrobacterium Phytochrome as an Enzyme for the Production of *ZZE* Bilins†

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Received November 26, 2004; Revised Manuscript Received April 20, 2005

ABSTRACT: Photoconversion of phytochrome from the red-absorbing form Pr to the far-red-absorbing form Pfr is initiated by a *Z* to *E* isomerization around the ring C−ring D connecting double bond; the chromophore undergoes a *ZZZ* to *ZZE* isomerization. In vivo, phytochrome chromophores are covalently bound to the protein, but several examples of noncovalent in vitro adducts have been reported which also undergo Pr to Pfr photoconversion. We show that free biliverdin or phycocyanobilin, highly enriched in the *ZZE* isomer, can easily be obtained from chromophores bound in a noncovalent manner to *Agrobacterium* phytochrome Agp1, and used for spectral assays. Photoconversion of free biliverdin in a methanol/HCl solution from *ZZE* to *ZZZ* proceeded with a quantum yield of 1.8%, but was negligible in neutral methanol solution, indicating that this process is proton-dependent. The *ZZE* form of biliverdin and phycocyanobilin were tested for their ability to assemble with Agp1 and cyanobacterial phytochrome Cph1, respectively. In both cases, a Pfr-like adduct was formed but the chromophore was bound in a noncovalent manner to the protein. Agp1 Pfr undergoes dark reversion to Pr; the same feature was found for the noncovalent *ZZE* adduct. After dark reversion, the chromophore became covalently bound to the protein. In analogy, the PCB chromophore became covalently bound to Cph1 upon irradiation with strong far-red light which initiated *ZZE* to *ZZZ* isomerization. *Agrobacterium* Agp2 belongs to a yet small group of phytochromes which also assemble in the Pr form but convert from Pr to Pfr in darkness. When the Agp2 apoprotein was assembled with the *ZZE* form of biliverdin, the formation of the final adduct was accelerated compared to the formation of the *ZZZ* control, indicating that the *ZZE* chromophore fits directly into the chromophore pocket of Agp2.

Phytochromes are photoreceptors of plants, bacteria, and fungi which absorb in the red to far-red region of the visible spectrum (*1*). Each phytochrome molecule carries one bilin chromophore, either biliverdin (BV),[1] phycocyanobilin (PCB), or phytochromobilin (PΦB), depending on the species and protein sequence. During an autocatalytical process, the chromophore becomes covalently attached to a cysteine residue. Biliverdin-binding phytochromes have a conserved cysteine in the N-terminus of the protein (around positions 5−25), which serves as the chromophore attachment site, whereas PCB- and PΦB-binding phytochromes have a conserved chromophore-binding cysteine within the so-called GAF domain of the protein, which lies around positions 250−330 (*1*). In principle, each of the exocyclic bilin double bonds can be in the *Z* or *E* configuration, whereas single bonds can adopt the syn or anti conformation (*2*). There are two long-lived, spectrally different forms in the photocycle of phytochromes, the red-absorbing Pr and the far-red-absorbing Pfr form. Phytochromes are synthesized in the Pr form, in which all three double bonds between the four pyrrole rings of the chromophore are in the *Z* configuration (*3*) (*ZZZ*; see Figure 1). Photoconversion into Pfr is triggered

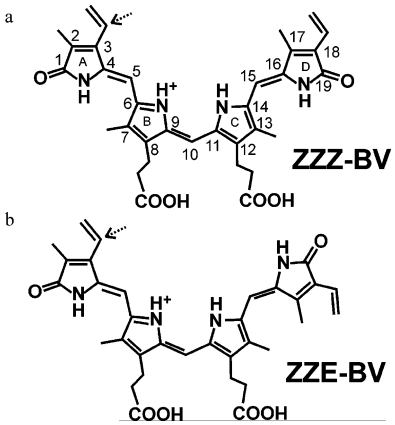

FIGURE 1: *ZZZ* (a) and *ZZE* (b) isomeric forms of BV. Isomerization from the *ZZZ* form to the *ZZE* form is the first step of phytochrome Pr to Pfr photoconversion. The ring A vinyl side chain (arrow) is involved in the formation of the covalent thioether bond with the apophytochrome protein. The bilin is drawn in a stretched conformation, which is the proposed conformation within the phytochrome chromophore pocket; free bilins adopt a rather helical conformation (*2*).

by a rapid *ZZZ* to *ZZE* isomerization around the ring C−ring D connecting methine bridge, and the trigger for the Pfr to Pr photoconversion is a rapid *ZZE* to *ZZZ* isomerization (*3*) (see Figure 1). Different models exist for the conformation of single bonds in the Pr and Pfr froms (see ref *4* and references therein). Plant phytochromes and phytochrome Agp1 (also termed AtBphP1) from *Agrobacterium tumefa-*

† This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 498, Teilprojekt B2.

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[1] Abbreviations: BV, biliverdin; PCB, phycocyanobilin; PΦB, phytochromobilin.

10.1021/bi047510g CCC: $30.25
Published on Web 05/21/2005

ciens undergo dark conversion from the Pfr to the Pr form in a time range of hours (*5*, *6*), whereas two bacterial phytochromes, BphP from *Bradyrhizobium* (*7*) and AtBphP2 (termed Agp2 here) from *A. tumefaciens* (*8*), undergo dark conversion from the Pr to the Pfr form. Thus, all phytochromes are light-dependent bilin *ZZZ* to *ZZE* and *ZZE* to *ZZZ* isomerases, and some phytochromes act also as light-independent *ZZE* to *ZZZ* isomerases and others as light-independent *ZZZ* to *ZZE* isomerases. In the case of PCB and PΦB, the ring A ethylidene side chain (C3=C3′) can be either in the *Z* configuration or in the *E* configuration (*9*, *10*), and it has been proposed that only the 3*E* isomer assembles with plant phytochrome (*10*).

Since bilins are synthesized in the *ZZZ* form, the thermodynamically stable configuration (*2*), free *ZZE* bilins are more difficult to obtain and to study. Stereoisomers of bilins have been produced by irradiating aluminum oxide-absorbed compounds (*11*). Under these conditions, either the C4=C5 or the C15=C16 group can undergo isomerization and the isomers can be purified by thin-layer chromatography. Production of *ZZE* isomers by other methods (*12*, *13*) has also been described. The PΦB and PCB chromophores of phytochrome and phycocyanin chromopeptides, respectively, could be converted to the *ZZE* form using mercaptoethanol (*14*).

Phytochrome assembly has so far only been studied with the *ZZZ* form (*15*, *16*). To test for chromophore−protein interaction, it might also be interesting to see if and in which way the *ZZE* chromophore is incorporated into a phytochrome protein. Such assays might provide further insight into the properties of the chromophore pocket, the assembly, and the photoconversion processes. Within a phytochrome molecule, the bilin absorbance maximum shifts by ∼50−70 nm to longer wavelengths upon *ZZZ* to *ZZE* isomerization, e.g., from 701 to 752 nm in Agp1 (*17*). If the protein is degraded or denatured in a neutral buffer, the spectral difference between both conformations is small, but under acidic conditions, the *ZZE* form absorbs at a lower wavelength than the *ZZZ* form (*18*, *19*). Where tested, the covalently bound phytochrome chromophore could be cleaved from the protein only by chromic acid treatment (*20*). However, several examples in which noncovalent chromophore−protein adducts are formed and undergo a more or less normal Pr−Pfr photocycle in vitro are known: (i) when the chromophore-binding cysteine of a biliverdin binding phytochrome is mutated to another amino acid (*6*), (ii) when PCB or PΦB is mixed with an apophytochrome which incorporates biliverdin in vitro (*17*), or (iii) when the covalent attachment site is blocked by a chemical agent such as iodoacetamide (*6*, *16*). In this paper, we used two strategies based on examples (i) and (ii) to obtain *ZZE* BV and *ZZE* PCB chromophores. These isomers were characterized with respect to their photochemical behavior and their incorporation into apophytochromes.

EXPERIMENTAL PROCEDURES

Bilin Chromophores. Biliverdin (BV) was purchased from Frontier Scientific (Carnforth, U.K.). Phycocyanobilin (PCB) was purified from dried *Spirulina platensis* cells by boiling methanolysis, phase partitioning, and high-performance liquid chromatography (HPLC) as described previously (*21*). Both bilins were stored in methanol stock solutions of 1−4 mM at −80 °C.

Protein Purification. Agrobacterium phytochrome Agp1 and the C20A mutant were expressed as His-tagged proteins in *Escherichia coli* and purified without a chromophore by Ni^{2+} affinity chromatography. The Agp1-C20A mutant was mixed with excess BV and subjected to size exclusion chromatography using a 2.5 cm × 100 cm Sephacryl S-300 (Amersham Bioscience) column, and the wild-type protein was purified in the same way without the chromophore (for details, see ref *6*). The size exclusion column was run in 50 mM Tris-HCl, 5 mM EDTA, and 150 mM NaCl (pH 7.8); this buffer was also used for protein mixing experiments. At this stage, the Agp1 (C20A) protein concentration was ca. 8 mg/mL (∼80 μM). *Synechocystis* phytochrome Cph1 was expressed as polyhistidine-tagged protein and purified by Ni affinity chromatography (for details, see ref *21*) and subjected to Sephacryl S-300 size exclusion chromatography as described for Agp1. The gene of *Agrobacterium* phytochrome Agp2 [also termed AtBphP2 (*8*)] including the stop codon was PCR-amplified from *Agrobacterium* genomic DNA and cloned into the BamHI and NdeI sites of the pET21b expression vector (Novagen) to obtain the pSA2 expression vector. The vector was transformed into *E. coli* strain DE3(BL21) (Novagen) for protein expression. The Agp2 protein of this strain is expressed without an affinity tag. Cell culture, induction of specific protein expression by IPTG, and extraction followed the protocol for Agp1. After extraction and centrifugation, the supernatant was subjected to ammonium sulfate (1.6 M) precipitation. After centrifugation at 20000*g* for 10 min, the precipitated proteins from a 100 mL culture were suspended in 2 mL of buffer [50 mM Tris-HCl, 5 mM EDTA, and 150 mM NaCl (pH 7.8)]. As determined from SDS−PAGE (*22*), Agp2 was the dominating protein in this fraction; the final Agp2 concentration was ∼4 mg/mL (∼40 μM), as judged by BV assembly. All proteins were stored at −80 °C until they were used.

Photoconversion and Spectral Measurements. All UV−vis spectral measurements were performed in a Uvikon 941 spectrophotometer (Kontron/Biotek, Milan, Italy). UV−vis spectra and photoconversion of free bilins were usually measured in a 95/5 methanol/HCl solution. For photoconversion of bilin adducts, the samples were irradiated in a quartz cuvette (4 mm × 10 mm) from above with a red light-emitting diode (emission maximum of 654 nm, half-bandwidth of 40 nm, and intensity of 32 $\mu mol\ m^{-2}\ s^{-1}$ at the top of the cuvette, measured with a PAR quantum SKP 215 light sensor, Skye Instruments, Powis, U.K.) for 2−5 min. The completeness of conversion was monitored by UV−vis spectroscopy. For the "Pr/Pfr absorbance change ratio", the absorbance value at the maximum of a Pr minus Pfr difference spectrum was divided by the absolute value at the minimum of the difference spectrum. The red light-emitting diode was also used to convert the *ZZE* into *ZZZ* bilins. For photoconversion of the Cph1 *ZZE*−PCB adduct into the *ZZZ* form, the cuvette was irradiated with a 780 nm laser diode (RLD78PZW2, Lasercomponents, Munich, Germany) at an output power of 70 mW from above, and the irradiated area was ca. 4 cm^2. The relative amount of Pfr under stationary conditions, φ, was obtained empirically. For Cph1 and other phytochromes, Pfr spectra have been calculated by the initial rate method (*23*). In the case of Agp1,

φ cannot be determined by the initial rate method (*24*) probably because non-photochemical Pfr to Pr conversion is too strong compared to the inefficient Pfr to Pr photoconversion (*6*). For the approach presented here, we calculated Pfr spectra for different φ values and compared these with the known Pfr spectrum of Cph1. The range limit of φ which gives Cph1-like Pfr spectra is only ca. 4%; other calculated spectra looked untypical. In Figures 2 and 6, the Pfr spectrum as calculated in this way is shown.

Extinction Coefficients and Photoconversion Quantum Yield. HPLC-purified *ZZE*-BV (see below) was used to obtain spectral parameters of this isoform. With the published extinction coefficient $\epsilon_{ZZZ-BV,698}$ of 30 600 M^{-1} cm^{-1} (*25*) and the spectra of pure *ZZE*- and *ZZZ*-BV (Figure 3b), the following extinction coefficients for BV in a methanol/HCl solution were obtained: $\epsilon_{ZZE-BV,622}$ = 43 000 M^{-1} cm^{-1} (absorbance maximum of *ZZE*-BV), $\epsilon_{ZZZ-BV,622}$ = 20 000 M^{-1} cm^{-1}, $\epsilon_{ZZE-BV,667}$ = $\epsilon_{ZZZ-BV,667}$ = 29 500 M^{-1} cm^{-1} (isosbestic point), $\epsilon_{ZZE-BV,698}$ = 15 500 M^{-1} cm^{-1}, and $\epsilon_{ZZE-BV,650}$ = 36 400 M^{-1} cm^{-1}. With these values, the relative content δ of *ZZE*-BV in a *ZZZ*/*ZZE* mixture can be calculated using the formula $\delta = (A_\lambda - cd\epsilon_{ZZZ,\lambda})/[cd(\epsilon_{ZZE,\lambda} - \epsilon_{ZZZ,\lambda})]$, where c is the total BV concentration, which can be obtained from the relation $c = A_{667}/\epsilon_{BV,667}$, and d is the measuring path length of the sample (here 1 cm). From the spectra, the absorbance values were taken at λ values of 622 and 698 nm for two independent calculations of δ. Both values were in good agreement (less than ±2%). To determine the kinetics of the *ZZE* to *ZZZ* photoconversion of free bilins in a methanol/HCl solution, actinic light from a halogen projector was directed onto the cuvette through a side window of the photometer. White light was filtered through a 650 nm DAL interference filter (Schott, Mainz, Germany) with a half-bandwidth of 12 nm, the light intensity was 13 μmol m^{-2} s^{-1} as measured with the Skye light sensor. The absorbance at 690 nm was measured continuously until no more photoconversion was obvious. The quantum yield Φ was calculated from the half-time of photoconversion $\tau_{1/2}$ using the formula $\Phi = \ln(2)/(2.3\tau_{1/2}N\epsilon)$ (*12*) with light intensity N and extinction coefficient ϵ_{ZZE-BV} at 650 nm.

Biliverdin Isomers. All steps were performed in darkness or under dim green safelight. The BV adduct of Agp1-C20A, purified by size exclusion chromatography after BV addition, was used unirradiated or after saturating red light irradiation had been carried out. One milliliter of the protein solution was mixed with 1 mL of a saturated ammonium sulfate solution [3.3 M in 50 mM Tris-HCl (pH 7.8)] and centrifuged at 20000*g* for 15 min. The supernatant was discarded and the residual liquid removed with filter paper. The protein pellet was mixed with 1 mL of methanol and mechanically ruptured. After a 15 min incubation on ice, the still insoluble protein was centrifuged at 20000*g* for 30 min; the bilin-containing supernatant was further used for spectral assays and chromophore assembly studies. Methanol solutions were usually concentrated under vacuum either to 100 μL or to dryness. In the latter case, the precipitate was redissolved in 50−100 μL of methanol. The concentration of this stock solution was determined by UV−vis spectroscopy.

Phycocyanobilin Isomers. For the preparation of PCB isomers, wild-type apo-Agp1 (final concentration of 10 μM) was mixed with excess PCB (final concentration of ca. 15 μM). Protein and bound PCB were separated from free PCB using NAP-10 desalting columns (Amersham Bioscience). Thereafter, PCB was separated from the protein as described for BV.

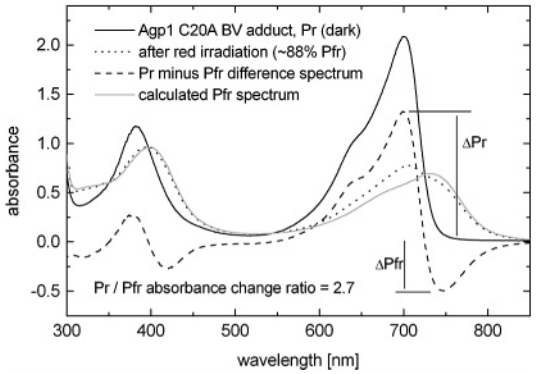

FIGURE 2: Absorbance and difference spectra of the BV adduct of the Agp1-C20A mutant: (—) before red irradiation and (···) after red irradiation. Since photoconversion is always incomplete, the latter sample contains both Pr and Pfr. The Pfr content of 88% was obtained empirically as described in Experimental Procedures; the Pfr spectrum which was calculated with this number is drawn as a solid gray line. The dashed black line is the Pr minus Pfr difference spectrum. The Pr/Pfr absorbance change ratio of 2.7, calculated from the ΔPr and ΔPfr values, is also given in the panel.

HPLC Analysis. For HPLC assays, BV isomers were separated on a C18 column (Ultrasep Es-pharm RP18E, 7 μm, 0.4 cm × 20 cm, Sepserv, Berlin, Germany) at a flow rate of 0.5 mL/min and monitored by the absorbance at 355 nm. The mobile phase consisted of acetonitrile, water, and acetic acid (40/58/2, v/v/v). Fractions of 0.5 mL were collected 15 min after injection (*ZZZ*-BV) and 17.7 min after injection (*ZZE*-BV) for spectral analyses. The purity was checked by a second HPLC run.

Assembly Studies. For chromophore assembly studies, Agp1, Agp2, or Cph1 (1 mg/mL = approximately 10 μM) was mixed in a measuring cuvette with 2−5 μM chromophore. Spectra were recorded immediately after mixing (t = 1 min) and at further time points as indicated.

RESULTS

Methanol Extraction of Biliverdin Isomers from Agp1-C20A and Initial Studies on ZZE−ZZZ Isomerization of Free BV. In the first step, we tested whether the biliverdin (BV) chromophore can be separated from the protein in either the Pr form or the Pfr form. To this end, we used the Agp1-C20A mutant (*6*) in which the chromophore-binding cysteine (*26*) is replaced with alanine. The BV adduct of this mutant also undergoes Pr to Pfr and Pfr to Pr photoconversion and Pfr to Pr dark reversion, as in the wild-type adduct. After assembly with excess chromophore, free BV was removed by size exclusion chromatography. At this step, the holoprotein was used unirradiated (*ZZZ*-BV control) or after it had been irradiated with red light for photoconversion into Pfr to obtain *ZZE*-BV. As in earlier work (*6*), the Pfr minus Pr difference spectrum of the C20A−BV adduct differed from that of the wild-type adduct. The maximum and minimum of Pr and Pfr were at 699 and 747 nm, respectively, and the Pr/Pfr absorbance change ratio was 2.7 (Figure 2); the corresponding values of the wild-type adduct are 700 nm, 752 nm, and 1.1, respectively (*17*). Judging from the absorbance spectrum, ca. 88% of the total phytochrome has been converted to Pfr (Figure 2). Biliverdin was removed

from the protein by ammonium sulfate precipitation and methanol extraction of the protein pellet. After centrifugation of the methanol solution, the still insoluble protein remained in the pellet, whereas BV was dissolved in the supernatant. Thereafter, the methanol solution was concentrated under vacuum to ca. 10% of the original volume. In general, spectra of free bilins were recorded in a methanol/HCl solution, since under these conditions there is a big spectral difference between the *ZZE* form and the *ZZZ* form, whereas the spectral difference is small in a neutral methanol or aqueous solution, as reported for PΦB and PCB chromopeptides (*18*, *19*) and free bilins (*11*). In addition, the extinction coefficient of the chromophore is higher in the protonated state, and bilins are more stable toward oxidation (*27*). It has been shown that within the phytochrome molecule, the chromophore is protonated in both the Pr form and the Pfr form (*28*, *29*). Thus, the protonation state within the protein is comparable with that of free chromophore in an acidic solution. Figure 3a shows UV−vis spectra of the BV released from unirradiated (dotted line) and red-irradiated Agp1-C20A (solid line). The absorbance maxima of BV in the red spectral region were 696 and 630 nm, respectively. When the latter sample was irradiated with red light, the maximum shifted to 696 nm; the shape of the spectrum (Figure 3a, dashed line) was identical with that of the *ZZZ* control. These measurements show that BV obtained from Agp1-C20A−Pfr adduct retained its *ZZE* configuration through separation and concentration and that irradiation in a methanol/HCl solution converts free *ZZE*-BV into the *ZZZ* form. We also irradiated neutral (only methanol) or basic *ZZE*-BV solutions (methanol with 50 mM NaOH); the relative fraction of the photoconverted chromophore was assayed after acidification (5% HCl). We found that a light treatment which converted 75% of *ZZE*-BV into *ZZZ*-BV under acidic conditions induced no significant conversion of *ZZE*- to *ZZZ*-BV under neutral or basic conditions. Thus, light-induced *ZZE* to *ZZZ* isomerization is a proton-dependent process. To test for the stability of the *ZZE* form in darkness, we incubated the sample in methanol with or without HCl for 16 h at 18 °C and measured the relative concentration of light-convertible bilin by difference spectroscopy. In both cases (with and without HCl), the content of *ZZE*-BV decreased to 75% after the incubation. Thus, thermal *ZZE* to *ZZZ* isomerization at room temperature is on the order of 1.6% per hour. Our results are in contrast with a report on the thermal stability of the C15═C16 *E* isomer of a similar bilin compound (*13*). Falk et al. (*13*) mentioned that upon the addition of acid, the *ZZE* isomer converted to the *ZZZ* form within 15 min in darkness. Another group reported that the *ZZE* isoform of phytochrome and phycocyanobilin chromopeptides is quite stable in acid (*18*), in harmony with our study, although these authors found that under alkaline conditions, rapid thermal *ZZE* to *ZZZ* conversion occurs, which we did not find. During our studies, we often stored *ZZE*-BV in methanol at −80 °C. The chromophore could be used for several weeks, but dark conversion was evident after prolonged storage for several months.

Purification of ZZE-BV by HPLC. We subjected a sample which contains *ZZE*- and *ZZZ*-BV at roughly equal concentrations to HPLC. Under conditions described in Experimental Procedures, two peaks were separated with elution maxima at 15 and 17.7 min. The 15 min fraction comprises

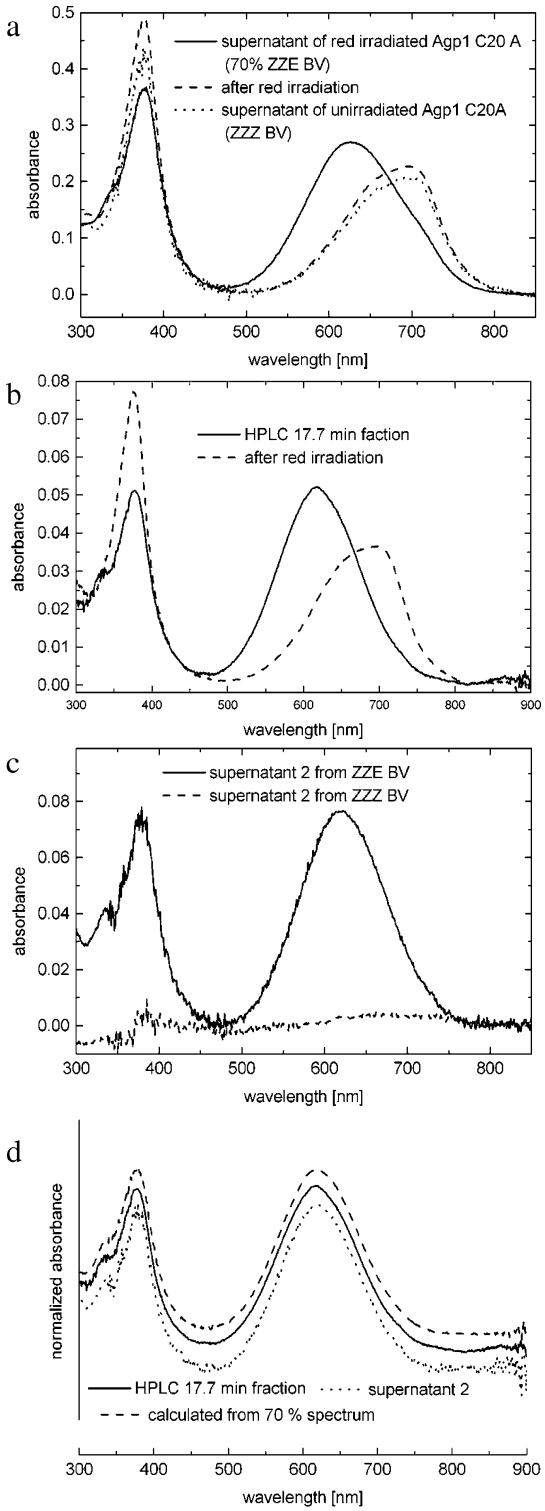

FIGURE 3: Spectra of free BV in a methanol/HCl solution (95/5). (a) Methanol supernatants of unirradiated (···) and red light-irradiated (—) Agp1-C20A. The latter sample, which contains ca. 70% *ZZE*-BV, was irradiated again to convert *ZZE*-BV to *ZZZ*-BV (- - -). (b) The 17.7 min HPLC fraction containing purified *ZZE*-BV before (—) and after (- - -) red irradiation. (c) Supernatant of a second methanol extraction. Either an *ZZE*-BV or an *ZZZ*-BV preparation as in panel a was mixed with wild-type Agp1. Thereafter, the noncovalently bound chromophore was extracted with methanol. This comparison shows that *ZZE*-BV is not covalently bound to the protein; the supernatant contains pure *ZZE*-BV. (d) Comparison of *ZZE*-BV spectra from panels b (—) and c (···), and the calculated *ZZE* spectrum from the spectrum of panel a (- - -). For ease of comparison, the spectra were normalized to A_{622} and drawn at different zero offsets.

ZZZ-BV, as shown by the UV−vis spectrum and by control runs with fresh BV, and the 17.7 min fraction contains purified *ZZE*-BV. Rechromatography showed that the compound was essentially free of *ZZZ*-BV (ca. 5% residual *ZZZ*-BV). The absorbance spectra of the purified *ZZE* sample in a methanol/HCl solution before and after red light-induced photoconversion into *ZZZ*-BV are shown in Figure 3b. From both spectra, optical parameters can be obtained which allow us to estimate the fraction of *ZZE*-BV in a *ZZE*/*ZZZ* mixture and to calculate the quantum yield of photoconversion. The absorbance maximum of pure *ZZE*-BV is at 622 nm, and thus at a lower wavelength than the maximum of the mixed isomers described above. The extinction coefficient $\epsilon_{ZZE-BV,622}$ equals 43 000 M^{-1} cm^{-1}, as calculated from the published $\epsilon_{ZZZ-BV,698}$ of 30 600 M^{-1} cm^{-1} (*25*). Since photoconversion proceeds only from *ZZE* to *ZZZ* and not in the opposite direction, the quantum yield Φ can be directly calculated from $\tau_{1/2}$ as outlined in Experimental Procedures. The Φ value was estimated to 1.8% and is thus higher than the Φ of the Pfr to Pr photoconversion of the BV−Agp1 adduct, which has been estimated to be 0.4% (*6*). It should, however, be mentioned that the low quantum yield for the Agp1−Pfr to Agp1−Pr photoconversion is untypical. The isosbestic point for *ZZE*- and *ZZZ*-BV is at 667 nm. From the extinction coefficient at this wavelength, the total BV concentration can be obtained directly also for isomer mixes. With the given concentration, the relative content of *ZZE*-BV can be estimated from an absorbance spectrum as described in Experimental Procedures. In the methanol supernatant from the photoconverted BV−C20A adduct (Figure 3a), this fraction was 70%. The normalized *ZZE* spectrum which was calculated from the spectrum of the mixed sample using this value is drawn together with the normalized spectrum of HPLC-purified *ZZE*-BV in Figure 3d. Within the resolution limits, both spectra were identical.

Assembly of ZZE-BV with Agp1. In the next step, a BV solution which contains 70% *ZZE*-BV and 30% *ZZZ*-BV was added to wild-type Agp1. We monitored by UV−vis spectroscopy whether and how the *ZZE* chromophore is incorporated into the protein (Figure 5). Immediately after mixing had taken place, a Pr peak and a Pfr-like shoulder appeared (Figure 5a). The relative content of the Pfr-like species was approximated from the first absorbance spectrum in the same way as for the Agp1−C20A adduct mentioned above to be ca. 70%, although the extinction coefficient of this species appears to be very low. During dark incubation, the magnitude of the "Pfr" shoulder decreased and that of the Pr peak increased as a result of dark conversion, a process well-known for Agp1 (*6*). For the calculation of a difference spectrum (Figure 5b, solid line), the $t = 1$ min spectrum was subtracted from the $t = 12$ min spectrum. In accordance with the low Pfr extinction coefficient, the Pr/Pfr absorbance change ratio of 3.8 was much higher than the corresponding value of Agp1 after the standard assembly with *ZZZ*-BV, which is ca. 1.1 (*17*). The maximum and minimum of the difference spectrum were at 702 and 745 nm, respectively (these values are comparable with Pr and Pfr maxima, respectively). Corresponding values for Agp1 assembled with *ZZZ*-BV are 700 and 752 nm, respectively (*17*). Thus, the spectral properties of Pfr obtained by incubation of Agp1 with *ZZE*-BV more closely resemble those of the photoconverted Agp1−C20A adduct, containing noncovalently bound

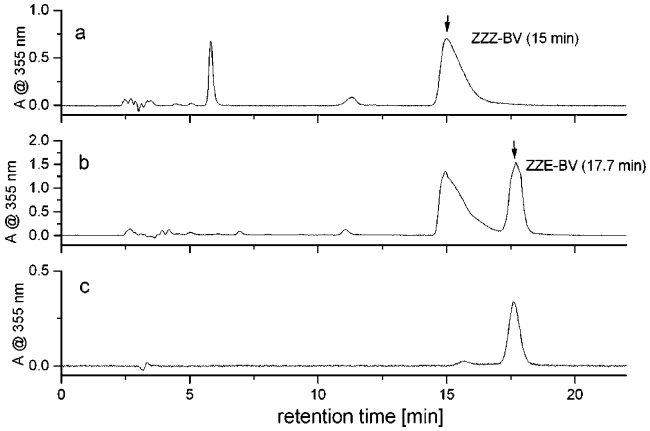

FIGURE 4: HPLC profiles, monitored at 355 nm, of *ZZZ*-BV as obtained from the commercial source, solubilized in methanol (a), a methanol supernatant of Agp1-C20A (b), and rechromatography of the 17.7 mL fraction (c). The 6 min peak in panel a is a contamination of unknown identity. The relative peak areas of the 15 and 17.7 min peaks in panel b are 72 and 50, respectively. Since the $\epsilon_{ZZE-BV,355}/\epsilon_{ZZZ-BV,355}$ ratio (the *ZZE*/*ZZZ* absorbance ratio at the measuring wavelength in the HPLC solution) is 0.7, the relative content of *ZZE*-BV in the applied sample was ca. 50%. The relative *ZZE* content is lower than that obtained with freshly prepared samples due to prolonged storage at −80 °C. After rechromatography of the 17.7 min fraction (c), a small peak of residual *ZZZ*-BV appeared around 15.5 min. The *ZZZ* position is slightly shifted because the sample was applied directly in the HPLC solution. The peak area ratio of the 15.5 and 17.7 mL peaks is 6/100. Considering the *ZZE*/*ZZZ* absorbance ratio at the measuring wavelength of 0.7, the content of *ZZZ*-BV was below 5%.

ZZE-BV (Figure 2), than those of the photoconverted wild-type adduct, containing covalently bound *ZZE*-BV. The UV−vis measurements clearly show that the *ZZE* chromophore is incorporated into the chromophore pocket and forms a Pfr-like adduct, which undergoes normal dark reversion, but the data also imply that the *ZZE* chromophore is not covalently attached to the protein. The covalent link between cysteine 20 of the protein and the ring A vinyl side chain is obviously formed after dark reversion into the *ZZZ* form. When the dark-reverted sample was irradiated with red light, the difference spectrum calculated from subsequent dark reversion was more similar with the *ZZZ*-BV-assembled holoprotein (Figure 5b, dashed line). The Pr/Pfr absorbance change ratio was 1.3, and the absorbance maximum and minimum were at 701 and 750 nm, respectively.

To check for covalent and/or noncovalent BV attachment, freshly assembled Agp1 adducts with *ZZZ*- and *ZZE*-BV were subjected to a second round of methanol extraction. Covalently bound chromophore should be retained in the protein pellet and should thus be absent from the supernatant. As expected, BV was released from the protein assembled with the *ZZE* chromophore but not from the *ZZZ* control sample (Figure 3c). This result confirms that *ZZE*-BV is bound in a noncovalent fashion to the protein, whereas covalent attachment of *ZZZ*-BV occurs quite rapidly. Because residual *ZZZ*-BV is removed by wild-type Agp1 from the *ZZE*/*ZZZ* mixture, the supernatant of the second extraction contains essentially pure *ZZE*-BV. The spectrum was again almost identical with the spectrum of HPLC-purified *ZZE*-BV (Figure 3d).

Phycocyanobilin Isomers and Assembly of ZZZ- and ZZE-Phycocyanobilin with Cph1. To see whether the binding mode of the *ZZE* chromophore is specific for Agp1 or

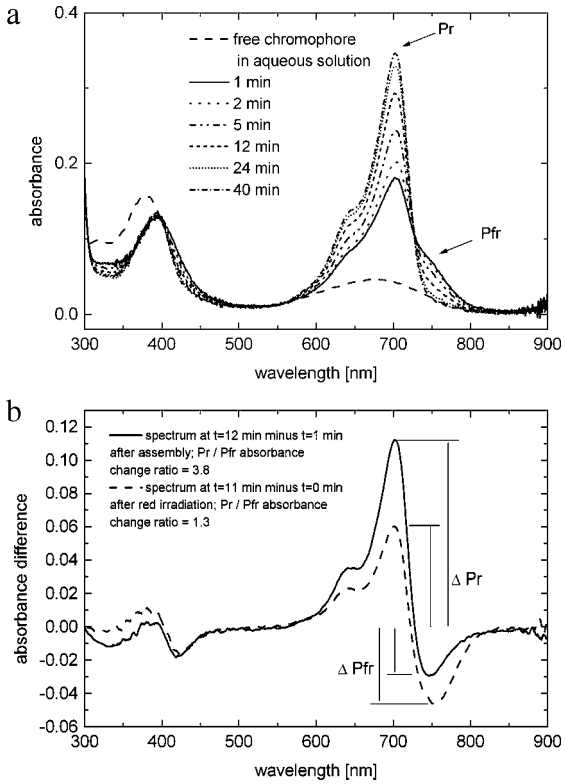

FIGURE 5: (a) Assembly of Agp1 with BV which has been extracted from red-irradiated Agp1-C20A (a mixture of *ZZZ*- and *ZZE*-BV). Absorbance spectra of the free chromophore and Agp1 upon mixing with the chromophore. The Pfr shoulder is formed immediately after the chromophore and protein have been mixed; over time as a result of dark reversion, the magnitude of the Pr peak increases and the magnitude of the Pfr shoulder decreases. (b) Dark reversion difference spectra of the same sample: (—) directly after *ZZE* assembly (spectrum at $t = 12$ min minus spectrum at $t = 1$ min) and (- - -) 40 min after *ZZE* assembly, in which the sample was converted by red light into Pfr (spectrum at $t = 11$ min minus spectrum at $t = 0$ min after the red irradiation).

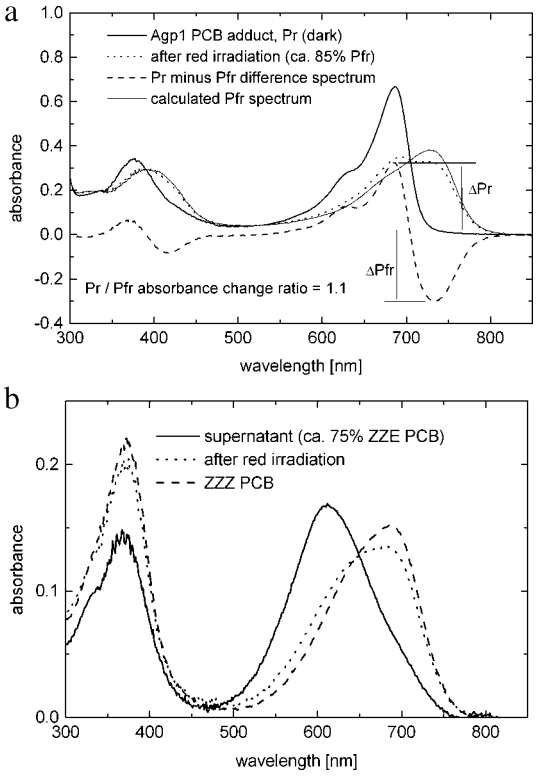

FIGURE 6: (a) Absorbance and difference spectra of the PCB adduct with wild-type Agp1 before (—) and after (•••) red light irradiation. The Pfr content was estimated to be 85% (see the text), and the Pfr spectrum which was calculated with this number is drawn with a gray line. The Pr/Pfr absorbance change ratio is calculated from the Pr minus Pfr difference spectrum (- - -). (b) Spectra of free PCB after methanol extraction of red-irradiated [predominately *ZZE*-PCB (—)] or unirradiated (- - -) PCB−Agp1 adduct measured in a methanol/HCl solution. The *ZZE* sample was irradiated by red light and measured again (•••).

whether other phytochromes behave similarly, we performed similar assembly experiments with cyanobacterial phytochrome Cph1, which incorporates PCB as a natural chromophore (*30*). To obtain *ZZE*-PCB, we used wild-type Agp1 for *ZZZ* to *ZZE* isomerization. Agp1 binds PCB efficiently but in a noncovalent manner; the adduct undergoes Pr to Pfr photoconversion (*6*). Figure 6a shows absorbance and difference spectra of a PCB−Agp1 sample which was used for later chromophore extraction. As in the case of BV above, we could obtain free PCB enriched in the *ZZE* isomer by methanol extraction, as demonstrated by UV−vis spectroscopy in a methanol/HCl solution (Figure 6b). Red light irradiation of the extracted *ZZE* sample also resulted in a spectral shift toward longer wavelengths, but the spectrum was still different from that of untreated *ZZZ*-PCB. We therefore assume that photoconversion of *ZZE*- to *ZZZ*-PCB is incomplete or that some spectrally different conformation or configuration is produced by the irradiation procedures. The spectral difference between the final species and untreated *ZZZ*-PCB could, for example, be related to a different isomeric state of the C3═C3′ ethylidene double bond (see ref *10*).

The spectral characteristics of PCB−Cph1 assembly differ from those of BV−Agp1 assembly, because during the assembly of Cph1, a noncovalent intermediate which absorbs at 690 nm is formed. A shift from 690 to 655 nm, the Pr absorbance maximum of Cph1, reflects the formation of the covalent bond (*16*). The shoulder of the 690 nm intermediate is seen in the control measurement with *ZZZ*-PCB as presented in Figure 7a. After Cph1 is mixed with a sample that contains predominantly *ZZE*-PCB, residual *ZZZ*-PCB leads to the formation of a Pr peak (Figure 7b). Also, a broad and flat shoulder in the longer wavelength region was observed. Since the abstracted spectrum which corresponds to the *ZZE* chromophore is different from that of free *ZZE*-PCB in aqueous solution, which has a broad peak with a maximum at 610 nm (data not shown), at least a fraction of the *ZZE* chromophore interacts with the protein and forms a Pfr-like adduct. Both the Pr absorbance and the absorbance at longer wavelengths increased during the subsequent 15 min. The increase in the level of Pr results from the transition of the noncovalently to covalently bound *ZZZ* chromophore (see control measurement in Figure 7a). The increase in the longer wavelength region results probably from an increase in the fraction of the (noncovalently) protein-bound *ZZE* chromophore. After an incubation time of 15 min, the sample was irradiated with strong far-red light (780 nm), which converted *ZZE*-PCB complexed with Cph1 into *ZZZ*-PCB. As a result, the Pr absorbance was increased. This immediate, strong increase was followed by a weak increase around 655 nm and a decrease in the 690 nm region during the subsequent 15 min. This spectral characteristic shows that the *ZZZ* chromophore which is now formed by far-red irradiation from *ZZE*-PCB undergoes the sequence from

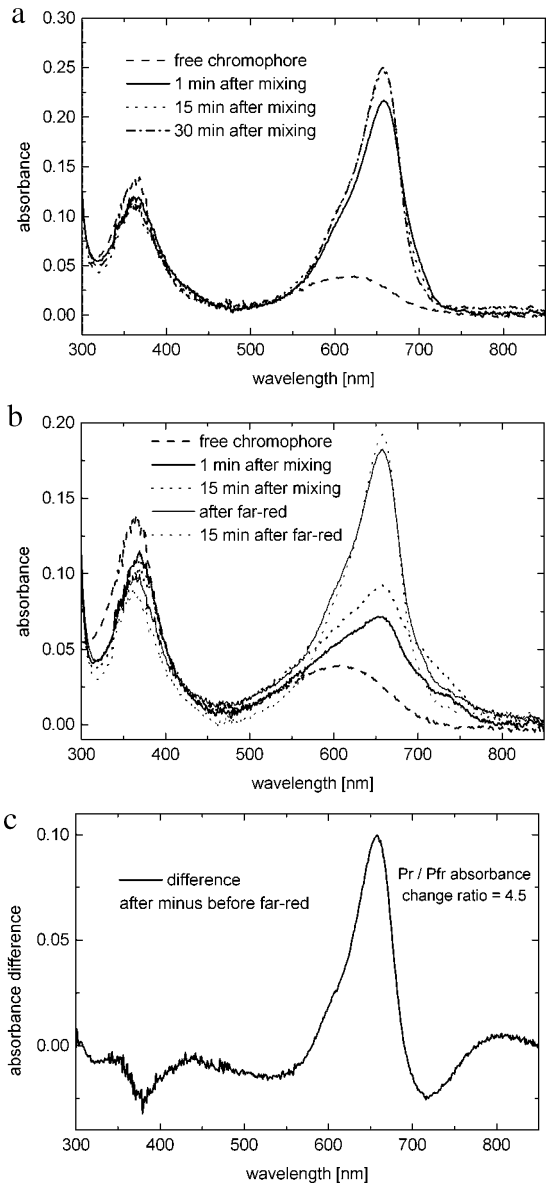

FIGURE 7: (a) Free *ZZZ*-PCB in buffer and after mixing with apo-Cph1 at 1, 15, and 30 min. The 690 nm intermediate is seen as a shoulder in the $t = 1$ min spectrum. (b) Free enriched *ZZE*-PCB in buffer and after mixing with apo-Cph1 at 1 and 15 min. Thereafter, the sample was irradiated with strong 780 nm far-red light to convert the *ZZE* form into the *ZZZ* form and again measured at 1 and 15 min. (c) Difference between the second and last absorbance spectra of panel b.

noncovalent to covalent binding, which in turn shows that before the irradiation, *ZZE*-PCB was noncovalently or improperly bound to the protein. From the difference spectrum, which was calculated from the absorbance spectrum before far-red irradiation and the spectrum 15 min after this irradiation, a Pr/Pfr absorbance change ratio of 4.5 was estimated; the position of the minimum (corresponding to the Pfr maximum) was at 728 nm. The absorbance change ratio for the normally assembled Cph1−PCB adduct lies between 0.8 (*23*) and 0.9 (*21*), and the Pfr maximum of difference spectra lies around 708 nm. All spectral features together indicate that *ZZE*-PCB binds in a noncovalent manner to Cph1, as was found above for the binding of *ZZE*-BV to Agp1. Methanol re-extraction of PCB from the *ZZE* adduct confirmed this assumption, although we could not obtain pure *ZZE*-PCB: in the *ZZZ* control, the methanol

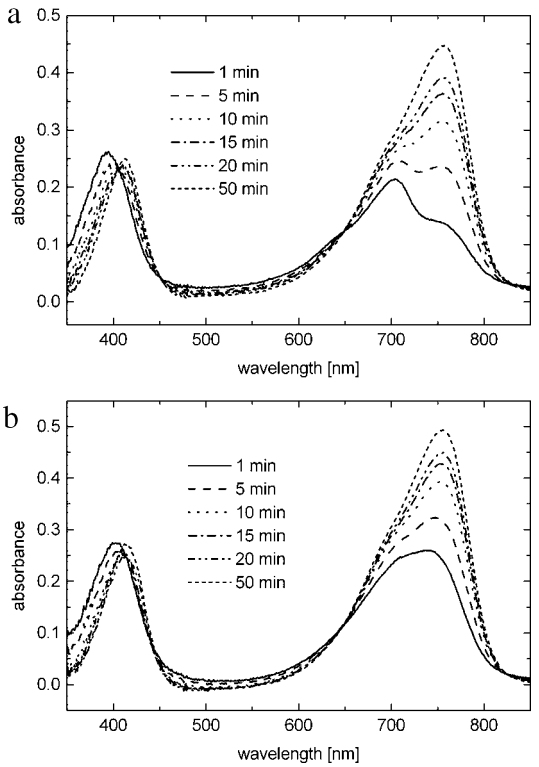

FIGURE 8: Reaction of Agp2 with *ZZZ*-BV (a) or with a 70/30 *ZZE*-BV/*ZZZ*-BV mixture (b). After the Agp2 extract had been mixed with BV, spectra were recorded at various times as indicated.

supernatant contained minor concentrations of the chromophore.

A third phytochrome, *Agrobacterium* phytochrome Agp2, was also tested for assembly with the two different BV isomers. Agp2 is exceptional: as opposed to Agp1, Cph1, and most other phytochromes, it undergoes Pr to Pfr dark conversion. This behavior is found after Pfr to Pr photoconversion and during chromophore assembly (*8*). After the apoprotein and *ZZZ*-BV have been mixed, a rather weak Pr peak appeared; prolonged incubation resulted in the formation of a stronger Pfr peak. From the pattern of spectral changes, it seems that isomerization of the chromophore takes place after Pr formation. When BV was offered in the *ZZE* form (70/30 *ZZE*/*ZZZ* mixture), the formation of Pfr was faster than in the experiment with *ZZZ*-BV (Figure 8b). Therefore, the *ZZE* chromophore fits directly into the chromophore pocket. Comparing panels a and b of Figure 8 shows that isomerization is not the only rate-limiting step in Pfr formation during chromophore assembly. The absorbance increase around 750 nm (from ca. 0.25 to ca. 0.5) after mixing with *ZZE*-BV shows that a slower reaction takes place. This ripening process is probably related to covalent attachment of the chromophore in its *ZZE* form. It thus seems that in Agp2, covalent attachment occurs in the *ZZE* form only.

DISCUSSION

In this paper, we show that free BV and PCB highly enriched in the *ZZE* isomer can be obtained from photoconverted Pfr of Agp1 or the C20A mutant of this protein by methanol extraction. Light irradiation induces a one-directional conversion of *ZZE*-BV to the *ZZZ* from in a HCl/methanol solution; a similar but incomplete photoreaction

was observed for PCB. In the case of BV, we also tested the photoreaction in neutral methanol or with added NaOH. In both cases, light did not lead to isomerization, indicating that for BV the photoreaction is a proton-dependent process, which to our knowledge has not been noticed before. From pure, HPCL-purified *ZZE*-BV, extinction coefficients were obtained. With these numbers, the quantum yield of the photoreaction could be calculated to 1.8%. The quantum yield of the corresponding PCB photoreaction was in the same range (data not shown). We used the bilin isomers for assembly studies with Agp1, Cph1, and Agp2. Agp1 and Cph1 represent the large group of phytochromes that have a stable Pr form (*6*, *23*), whereas Agp2 represents a yet small group of phytochromes that convert from Pr to Pfr in darkness (*8*). In both Agp1 and Cph1, the *ZZE* chromophore binds to the protein and forms a characteristic adduct absorbing in the far-red spectral region, but the species produced in this way has a low extinction coefficient. The properties of this species, dark reversion into Pr in the case of Agp1 and photoconversion into Pr in the case of Cph1, showed that indeed a Pfr-like adduct was formed in both cases. The *ZZE* chromophore bound in a noncovalent fashion to the Agp1 and Cph1 proteins. It is obvious from our measurements and other data (*6*, *16*, *17*, *21*) that the extinction coefficient of "noncovalent Pfr" is rather small. In addition, the absorbance maximum of the *ZZE*-PCB−Cph1 adduct is shifted to longer wavelengths compared to that of covalent Pfr. Since Cph1 and Agp1 are quite unrelated to each other (*1*), these findings also probably hold for other phytochromes, e.g., from plants. Why does covalent coupling take place only with *ZZZ* and not with *ZZE* bilins, although both isoforms are incorporated into the chromophore pocket of the respective apophytochrome proteins? One explanation could be that the lyase reaction desires a particular electron configuration of the chromophore, which would not be given with the *ZZE* isomer. However, Cph1 forms a covalent adduct with PEB (*21*, *31*), which lacks the double bond between rings C and D, showing that the ring D electron system has no direct impact on covalent attachment. A more likely explanation is that the ring A vinyl side chain of *ZZE*-BV and the ring A ethylidene side chain of *ZZE*-PCB, which are involved in covalent bond formation (*17*), are spatially displaced, because covalent and noncovalent chromophores are under different tensions within the chromophore pocket. Ultrafast absorbance measurements on the picosecond time scale have shown that the rise time of light-induced bilin isomerization within the Agp1 protein is not affected by the mode of chromophore attachment (covalent vs noncovalent) (*32*). Thus, differential tension is probably generated during a later step of the Pr to Pfr photoconversion. Photocycle measurements on the microsecond to millisecond time scale revealed large kinetic differences between a covalent and a noncovalent adduct of cyanobacterial phytochromes (*33*). On the basis of infrared spectroscopy with plant phytochromes and density functional theory calculations, it has been proposed that during Pr to Pfr photoconversion, the initial *ZZZ* to *ZZE* isomerization is followed by a syn to anti rotation around the single bond between rings A and B (*4*). It could be that in the noncovalent *ZZE* adducts this rotation is incomplete. In this case, the vinyl or ethylidene side chain would be too far from the reacting cysteine residue. Although, in vitro, noncovalent phytochrome adducts undergo Pr to Pfr photoconversion, the covalent attachment of the chromophore is important for the function of phytochromes in vivo, since almost all known phytochrome proteins (to date more than 130) have a cysteine residue which is homologous with either the plant PΦB binding site or the BV binding site of Agp1 (*1*).

In the case of Agp2, *ZZE*-BV yielded an adduct with a normal Pfr spectrum. Moreover, Pfr formation was more rapid than with *ZZZ*-BV. The conversion of the latter chromophore into the *ZZE* form is an obvious rate-limiting step in the assembly of Agp2; this step is circumvented if the *ZZE* chromophore is directly offered. Our measurements with *ZZE*-BV show that there is another rate-limiting step in the assembly process of Agp2, which is probably related to the formation of the covalent link. In addition, the results show that the isomerization of the chromophore does not have to take place within the chromophore pocket of the protein and that the protein adjusts its conformation without forming photocycle intermediates. The discovery of phytochromes that convert in darkness into Pfr was surprising, because the *ZZZ* form of free bilins is thermodynamically more stable than the *ZZE* form. Therefore, the energy for the dark conversion of Agp2 cannot be stored in the chromophore alone. Covalent chromophore attachment might be one prerequisite for the change in energy relation in Agp2. It will be interesting to study the role of covalent bond formation for the assembly and photoconversion process of Agp2, e.g., by analyzing a site-directed mutant or by blocking studies.

ACKNOWLEDGMENT

We thank Sabine Artelt for technical help with the cloning of the Agp2 expression construct, Mansour Assadi, who purified the Agp1-C20A mutant during a practical course, and Magdalena Swiatecka for help with PCB purification. We also thank both reviewers for their helpful comments.

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BI047510G