Sterically Locked Synthetic Bilin Derivatives and Phytochrome Agp1 from Agrobacterium tumefaciens Form Photoinsensitive Pr- and Pfr-like Adducts*

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Phytochrome photoreceptors undergo reversible photoconversion between the red-absorbing form, Pr, and the far-red-absorbing form, Pfr. The first step in the conversion from Pr to Pfr is a Z to E isomerization around the C15=C16 double bond of the bilin chromophore. We prepared four synthetic biliverdin (BV) derivatives in which rings C and D are sterically locked by cyclizing with an additional carbon chain. In these chromophores, which are termed 15Za, 15Zs, 15Ea, and 15Es, the C15=C16 double bond is in either the Z or E configuration and the C14–C15 single bond in either the syn or anti conformation. The chromophores were assembled with Agrobacterium phytochrome Agp1, which incorporates BV as natural chromophore. All locked BV derivatives bound covalently to the protein and formed adducts with characteristic spectral properties. The 15Za adduct was spectrally similar to the Pr form and the 15Ea adduct similar to the Pfr form of the BV adduct. Thus, the chromophore of Agp1 adopts a C15=C16 Z configuration and a C14–C15 anti conformation in the Pr form and a C15=C16 E configuration and a C14–C15 anti conformation in the Pfr form. Both the 15Za and the 15Ea adducts absorb in the blue region of the visible spectra. All chromophore adducts were analyzed by size exclusion chromatography and histidine kinase activity to probe for protein conformation. In either case, the 15Za adduct behaved like the Pr and the 15Ea adduct like the Pfr form of Agp1. Replacing the natural chromophore by a locked 15Ea derivative can thus bring phytochrome holoprotein in the Pfr form in darkness. In this way, physiological action of Pfr can be studied in vivo and separated from Pr/Pfr cycling and other light effects.

Phytochromes are photoreceptors of plants, bacteria, and fungi with a bilin chromophore that absorb light in the red and far-red region (1). The natural chromophore differs between species: land plants use phytochromobilin (2) and cyanobacteria use phycocyanobilin (3), whereas other bacteria use biliverdin (BV) (4). The natural chromophore attaches covalently to a cysteine residue by an autocatalytic process, although in vitro adducts with non-covalently bound chromophores may also be spectrally active (5, 6). Agrobacterium phytochrome Agp1, which is used in the present study, belongs to the BV binding phytochromes and attaches its chromophore to Cys-20 (5, 7) via the ring A vinyl side chain of BV (8). Upon assembly, the red absorption maximum (λmax) of the chromophore shifts toward a longer wavelength, accompanied by an absorbance increase, to form the red-absorbing Pr. Light absorption of Pr initiates photoconversion into the Pfr form, which results in a further ~50–70-nm red shift of λmax. Light absorption of Pfr initiates the reverse photoconversion into Pr. For plant phytochromes it has been shown that the first step of the Pr to Pfr photoconversion is a Z to E isomerization of the chromophore around the C15–C16 double bond (9), which occurs in the picosecond time scale (10, 11). Isomerization is followed by spectral changes in the microsecond and millisecond time scale (12). During photoconversion, the chromophore also undergoes movements around the exocyclic single bonds. In principle, each single bond can adopt either the syn or anti conformation (13). Vibrational spectroscopy gained indirect insight into the conformation of the phytochrome chromophore in the Pr, Pfr, and intermediate states, but the data were not unambiguous and have been interpreted in different ways (14–17). For example, it has been proposed that the formation of Pfr is accompanied by a syn/anti rotation around the C14–C15 single bond (14). More recently, interpretation of Resonance Raman spectra by density functional theory calculations proposed that the C14–C15 single bond is in the anti conformation throughout the entire photocycle and that the C5–C6 single bond rotates from anti to syn upon conversion from Pr to Pfr (17).

The Pr/Pfr photocycle is associated with conformational changes of the protein that have been measured by size exclusion chromatography, CD spectroscopy, or limited proteolysis (18–20). Most bacterial phytochromes are histidine kinases in which kinase activity is modulated by light-induced conformational changes of the protein. Kinase activity is generally stronger in the Pr and weaker in the Pfr form, although other phosphorylation patterns have also been found (4, 5, 21, 22). Conformational changes of the protein also trigger different specific phosphorylation events of plant phytochromes (23, 24) and their association with interacting protein partners such as PIF3 (25).

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To understand better how the structure of the chromophore is linked with the conformation of the protein, we assembled Agrobacterium Agp1 with different synthetic sterically locked chromophores. Synthetic bilin derivatives have been used to test for the role of side chains and to study photoconversion and physiological action of plant phytochromes (8, 26–28). The chromophores used in the present work include four BV derivatives in which the rings C and D are fixed by an additional carbon chain in either the Z-anti, Z-syn, E-anti, or E-syn configuration/conformation. The adducts were analyzed by UV-visible spectroscopy and protein assays that allow testing for the conformational difference between Pr and Pfr proteins.

EXPERIMENTAL PROCEDURES

Synthesis of Bilin Derivatives—Biliverdin was purchased from Frontier Scientific (Carnforth, UK). Synthesis of 15EtBV was performed according to the protocols published for phytochromobilin, phycocyanobilin, and phycoporphyrinobilin derivatives (29–32). 15Za and 15Ee BV derivatives were prepared as reported (33, 34). Syntheses of 15Zs and 15Es will be published elsewhere. Bilin stock solutions of ~1 mM were prepared in dimethyl sulfoxide (Me$_2$SO) and stored at −80 °C.

Expression and Purification of Agrobacterium Phytochrome Agp1—An Escherichia coli expression vector termed pAG1 was used for full-length Agp1. pAG1 encodes for the Agp1 protein with a C-terminal polyhistidine tag for Ni$^{2+}$ affinity purification (5). Another vector, pAG1-M15, which encodes for the N-terminal 504 amino acids of Agp1 followed by 6 C-terminal histidine residues, was constructed. This vector was produced by PCR amplification using pAG1 as template followed by ligation. Correct cloning was confirmed by DNA sequencing. The expressed protein is termed Agp1-M15. For expression and purification of Agp1 and Agp1-M15 apoproteins, we followed the same procedure as described earlier (35). In brief, E. coli cells harboring the expression plasmid were grown in rich broth medium. Specific protein expression was induced by isopropyl-1-thio-$\beta$-D-galactopyranoside, and the cells were disrupted with a French pressure cell. Soluble proteins were purified by Ni$^{2+}$ affinity chromatography and by preparative size exclusion chromatography (SEC). The SEC was performed in “basic buffer” (300 mM NaCl, 50 mM Tris/Cl, 5 mM EDTA, pH 7.8). After SEC, the apoprotein concentration was ~15 mg/ml. At this stage, the protein was stored at −80 °C.

Assembly and UV-visible Spectroscopy—UV-visible spectra were recorded as described before (8) at 18 °C for assembly studies, the apoprotein was diluted with basic buffer to a final concentration of 10 μM, as judged from absorbance at 280 nm, and pipetted into a measuring cuvette. After addition of 1 mM dithiothreitol or 1 mM Tris(2-carboxyethyl)phosphine hydrochloride as Cys reducing agent, chromophore was added from the Me$_2$SO stock solution to a final concentration of ~5 μM; the sample was mixed rapidly for 30 s. Immediately after mixing, a spectrum was recorded from 900 to 250 nm; the duration of the scan was ~1 min. The first spectrum was labeled “1 min.” Further spectra were recorded at least until no spectral changes were obvious. To analyze whether chromophores are covalently bound to the protein, apoprotein and chromophore were mixed as above and incubated overnight in darkness at 18 °C. Thereafter, SDS was added to a final concentration of 1% and the sample was incubated again for 10 min. The mixture was passed over a desalting column as described earlier (5, 8). From a comparison of absorption spectra before and after column separation, the amount of covalently bound chromophore can be estimated. In some cases, covalent binding was also tested 5 min after mixing. To analyze non-covalent binding, the apoprotein was incubated with 2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) for 30 min, which blocks the covalent binding site (5). Because DTNB also reacts with the reducing agent that was present in the apoprotein solution, rather high concentrations of the yellow 5-thio(2-nitrobenzoic acid) product, which has a λ$_{max}$ of 412 nm, were formed. Blocked apoprotein was therefore passed over a desalting column to reduce the concentration of this compound. Thereafter, chromophore was added to the protein and a part of the sample was passed again over a desalting column as above. Before recording of UV-visible spectra of the samples, 1% SDS was added to reduce the impact of the protein on the chromophore spectrum. Residual 5-thio(2-nitrobenzoic acid) product, which was still present in the sample before the final desalting column separation, slightly increased the absorbance in the blue region. For photoconversion, the samples were irradiated for 5 min with either red light from a 655-nm light emitting diode (50 μmol m$^{-2}$ s$^{-1}$) or white light from a light bulb (100 μmol m$^{-2}$ s$^{-1}$).

Analytical Size Exclusion Chromatography—The Agp1-M15 apoprotein (10 μM) was mixed with excess molar amounts of chromophore (~15 μM) and incubated for ~3 h. The BV and 18EtBV adducts were used either unirradiated (Pr) or after saturating red irradiation (Pfr). SEC was carried out on a Superdex 200 HR 10/30 (Amersham Biosciences) column run at 500 μl/min with 50 mM Tris/Cl, 150 mM NaCl, 5 mM EDTA, pH 7.8. The system was calibrated with marker proteins, cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), α-amylase (299 kDa), and apoferritin (443 kDa) (Sigma). The elution was monitored at 280 nm.

Autophosphorylation—The same irradiation and concentration conditions were chosen for autophosphorylation assays. Phosphorylation was performed as described previously (5). The incubation time was 5 min.

RESULTS

Chromophore Assembly—Six different chromophores were used for assembly with Agrobacterium phytochrome Agp1: biliverdin (BV), 18,18′-dihydrobiliverdin (18EtBV), 15Z-anti-18EtBV (15Zs), 15Z-syn-18EtBV (15Za), 15E-anti-18EtBV (15Es), and 15E-syn-18EtBV (15Ee). In the latter four chromophores, the rings C and D are sterically locked by an additional carbon chain in one of four possible stereoisomers: the C15=C16 double bond is in either the Z or E configuration and the single bond in either the syn or anti conformation (see Fig. 1). Biliverdin, the natural chromophore of Agp1 (5), and 18EtBV served as controls. Both chromophores form a covalent bond with the apoprotein and produce adducts that show typical red/far-red reversible photoconversion (8). Phytochrome
chromophore assembly can be followed by UV-visible spectroscopy. Phytochrome chromophores have two absorption peaks in the blue and red region. Upon incorporation into the chromophore pocket, the red peak shifts to longer wavelengths and the absorbance increases above the level of the blue absorption band. The non-covalent BV adduct of Agp1 has the same $\lambda_{\text{max}}$ as the covalent adduct, but the extinction coefficient of the former is lower (5, 8). Therefore, the transition from the non-covalent to the covalent state during BV-Agp1 assembly is characterized by an absorbance increase. In our present study, the absorbance increase was completed between $t = 1$ min and $t = 5$ min after mixing chromophore and protein (Fig. 2a). The Pr $\lambda_{\text{max}}$ in the red spectral region was at 701 nm (Table I). Assembly with 18EtBV was also completed between 1 and 5 min, and the Pr adduct had a red $\lambda_{\text{max}}$ at 691 nm (Fig. 2b, Table I). The absorption maximum is blue shifted compared with that of BV-Agp1, because the conjugation system of 18EtBV is shorter than that of BV by the lack of a vinyl group at the C18 position (Fig. 1). With 15Za, a qualitatively similar assembly pattern was observed as with BV and 18EtBV (Fig. 2c). The spectrum of the adduct has a Pr-like form with two major absorption maxima in the red and blue, and a red $\lambda_{\text{max}}$ appeared at 714 nm (Table I). The absorbance bands of this adduct are sharper compared with the Pr form of the BV and 18EtBV adducts. Besides the two major absorption bands in the red and blue regions, phytochromes have the third absorption band in the red spectral region, which lies at shorter wavelengths than the red absorption maximum and appears as a shoulder in the spectra of the BV and 18EtBV adducts (Fig. 2, a and b). In the 15Za adduct, this band appears as the third peak with a $\lambda_{\text{max}}$ at 649 nm (Table I). Another spectral difference between the three adducts is the absorbance ratio between the major red and blue absorption bands, which increases from BV over 18EtBV to 15Za (Table I).

Both 15Za and 15Es chromophores gave spectrally unusual adducts. Immediately after mixing chromophore and protein, the red absorption band was lost (Fig. 2, d and f). These data imply that the interaction between chromophore and protein is rapid and completed within 1 min. The adducts have only a blue absorption band with a $\lambda_{\text{max}}$ at 430 nm for 15Zs and 433 nm for 15Es.

Immediately after mixing protein and 15Ea (Fig. 2e), an absorption band arose with a $\lambda_{\text{max}}$ at 727 nm. This band increased steadily and shifted to 739 nm during prolonged incubation up to $\sim$140 min. Thereafter, no spectral changes were observed. Because the first spectrum after mixing differs from the spectrum of the free chromophore, the chromophore-protein interaction is also rapid. The slow spectral changes indicate an enduring second reaction, most likely the formation of the covalent bond. The spectrum of the final adduct is similar to the calculated Pfr spectra of the BV and, more particularly, the 18EtBV adducts (Fig. 3, a and b), which have a $\lambda_{\text{max}}$ at 747 and 740 nm, respectively (Table I). The positions of the major red and blue absorption maxima of 18EtBV-Pfr and 15Ea adducts are 1 and 10 nm separate, respectively. The position of the side shoulder is also comparable (3 nm separate), and the red/blue absorbance ratios are similar (Table I).

**Photoconversion**—It has been shown that the first step in the Pr to Pfr photoconversion of plant phytochromes is a Z to E isomerization around the C15–C16 double bond (9). Because of the spectral similarities, this mechanism is most likely universal for all phytochromes, although direct experimental evidence is lacking. If Agp1 follows the same mechanism, all adducts with locked chromophores should remain stable in the light. Indeed, we did not observe any spectral changes upon irradiation with either red or white light (Fig. 3, c–f) under conditions that induced saturating conversions of the BV and 18EtBV adducts (Fig. 3, a and b).

**Covalent Attachment and Strength of Chromophore-Protein Interaction**—In the next step, we tested whether the chromophores are covalently bound to the protein. After mixing chromophore and protein and overnight incubation, SDS was added to dissociate non-covalent interactions, and the solutions were subjected to desalting columns. In this way, the protein is separated from non-bound chromophore. By comparing normalized UV-visible spectra before and after this separation, the fraction of covalently bound chromophore can be estimated (5, 8). It turned out that all chromophores bound quantitatively in a covalent manner to the Agp1 protein (Fig. 4). Additional experiments were performed with BV and 15Ea in which the incubation time (before SDS addition) was only 5 min. Although under these conditions BV was still quantitatively
bound (data not shown), the fraction of bound 15Ea was rather low (Fig. 4, inset). This is in line with the above assumption that the spectral changes observed during 15Ea assembly reflect the formation of the covalent bond.

We performed a similar desalting column test after blocking the chromophore binding site by DTNB. In this case, column separation was performed in the native state without SDS. (SDS was added before measuring UV-visible spectra both before and after column separation.) In this way, the strength of the chromophore-protein interaction was tested without the bias of the covalent bond. In all six cases the chromophores remained bound to the protein during the separation (Fig. 5). Thus, all chromophores interact strongly with the protein.

**Protein Conformation, Size Exclusion Chromatography—** The mobility of proteins subjected to SEC is dependent on the shape and quaternary structure of the molecules. In previous analyses with cyanobacterial phytochrome Cph1, we found SEC mobility differences between Pr, Pfr, and the apoprotein (35). Pr/Pfr differences were more pronounced for N-terminal fragments of Cph1 that lack the C-terminal histidine kinase domain (20, 36). We subjected full-length Agp1 apoprotein as well as Pr and Pfr of the BV adduct to SEC but found no significant mobility differences between these samples. We also cloned and purified the N-terminal chromophore module, termed Agp1-M15, which ranges from amino acid 1 to 504 and lacks the C-terminal histidine kinase module. The mobility of apoprotein and the Pr and Pfr forms of the BV adduct of Agp1-M15 on SEC were different, these values corresponding to apparent molecular sizes of 120, 110, and 145 kDa, respectively (Fig. 6a). The results with Pr and Pfr of the 18EtBV adduct were comparable with those of the corresponding BV adduct (Fig. 6b). The 15Za adduct eluted with an apparent molecular mass of 115 kDa (Fig. 6c), in between the Pr forms of BV-Agp1-M15/18EtBV-Agp1-M15 and the Agp1-M15 apoprotein. The mobilities of the 15Zs and 15Es adducts were identical to that of the apoprotein (Fig. 6, d and f), whereas the mobility of the 15Ea adduct was comparable with that of the Pfr form of the BV and 18EtBV adducts (Fig. 6e). Because the molecular mass of the Agp1-M15 monomer is 55,830 Da, we assume that in all cases the Agp1-M15 protein formed homodimers and that the differences in retention time result from changes of the shape of the molecule.

**Histidine Kinase—** The histidine kinase autophosphorylation activity of Agp1 is light regulated: upon photoconversion from

### Table 1

| Chromophore; Pr or Pfr | λ<sub>max</sub> in blue spectral region | λ<sub>max</sub> of side shoulder/side peak in red spectral region | λ<sub>max</sub> in red spectral region | Absorption ratio red maximum/blue maximum |
|------------------------|--------------------------------------|-------------------------------------------------|----------------------------------|----------------------------------------|
| BV; Pr                 | 393                                  | 651                                             | 701                             | 2.3                                    |
| BV; Pfr                | 413                                  | 702                                             | 747                             | 1.4                                    |
| 18EtBV; Pr             | 382                                  | 639                                             | 691                             | 3.1                                    |
| 18EtBV; Pfr            | 406                                  | 668<sup>a</sup>                                 | 740                             | 2.0                                    |
| 15Zα                   | 385                                  | 649                                             | 714                             | 10.4                                   |
| 15Zs                   | 430                                  |                                                  |                                  |                                        |
| 15Ea                   | 396                                  | 683                                             | 739                             | 1.8                                    |
| 15Es                   | 433                                  |                                                  |                                  |                                        |

<sup>a</sup> This shoulder is only weakly pronounced; the position could not be determined precisely.

**Fig. 3. Effect of irradiation on Agp1 adducts.** The BV (a), 18EtBV (b), 15Za (c), and 15Ea (e) adducts were irradiated with red light and the 15Zs (d) and 15Es (f) adducts with white light. The spectrum before and after red irradiation is given in each panel. Only the BV and 18EtBV adducts underwent photoconversion; adducts with locked chromophores were stable in the light. In the case of the BV and 18EtBV adducts, the Pfr levels after saturating red irradiation were estimated to be 90 and 88%, respectively. The spectra of pure Pfr were calculated and also presented in the panels (a, b). Protein and chromophore concentrations were similar as in Fig. 2; the spectra were normalized to A<sub>280</sub> of 1.
Pr to Pfr, the activity of Agp1 becomes reduced. We measured autophosphorylation of our different Agp1 adducts to test for the impact of the locked chromophores on kinase activity (Fig. 7). The mean activity of BV-Agp1-Pr was set to 100%. The mean activity of the apoprotein was 130%. The slightly stronger activity of the apoprotein as compared with the Pr form of the adduct is in line with previous studies (5), although in our present studies the difference was not significant because of variations among different sets of experiments. Upon conversion into Pfr, the mean activity was diminished to 19%, again in accordance with previous studies (5). The data of the 18EtBV adduct were comparable with those of the BV adduct: the mean activity of Pr was 110% and that of Pfr 18%. The mean activity of the 15Za adduct was 130% and thus comparable with that of the apoprotein, but differences between 15Za and the Pr forms of the BV and 18EtBV adducts were not significant. The mean activity of the 15Ea adduct was only 20% and thus comparable with the activity of Pfr of BV and 18EtBV adducts of Agp1. It should be mentioned that after 15Ea assembly all holoprotein molecules adopt the Pfr-like state, whereas in the case of red irradiated BV and 18EtBV adducts, only ~90% Pfr were obtained. The phosphorylation data of the 15Ea adduct imply that Pfr has indeed a residual phosphorylation activity. Such a conclusion could hardly be

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**FIG. 4. Covalent chromophore attachment of BV (a), 18EtBV (b), 15Za (c), 15Zs (d), 15Ea (e), and 15Es (f) adducts.** After mixing chromophore and protein, SDS was added to dissociate non-covalent bindings. Samples were incubated overnight except in panel e (inset), where the incubation time was 5 min. Thereafter, an aliquot of the sample passed through a desalting column. Normalized spectra (A<sub>280</sub> = 1) before and after the separation are given in each panel. The comparison of both spectra shows that in all cases except panel e (inset), the chromophore elutes with the protein and is thus covalently bound to the protein. The spectral difference between the free chromophore in assembly buffer (Fig. 2) and the data presented here is related to SDS, as shown by control measurements. The protein concentration was always 10 μM and the chromophore concentration ~5 μM.

**FIG. 5. Non-covalent chromophore attachment of BV (a), 18EtBV (b), 15Za (c), 15Zs (d), 15Ea (e), and 15Es (f) adducts.** The covalent chromophore binding site of the protein was blocked by DTNB and the sample passed over a desalting column to remove free DTNB. After mixing chromophore and blocked protein, an aliquot of the sample was passed through a desalting column. Before measuring UV-visible spectra, SDS was added to diminish the impact of the protein on the chromophore spectrum. Normalized spectra (A<sub>280</sub> = 1) before and after the separation are given in each panel. The comparison of both spectra shows that in each case the chromophore migrates with the protein and thus interacts strongly with the blocked protein. After the reaction of DTNB with free sulphydryl groups, a product with an absorption maximum at 412 nm is formed. This product is present in the samples "before separation" but lost during desalting column separation. For this reason, the absorbance around 412 nm is always higher in the samples before separation.
Pr, after dark incubation; Pfr, after saturating red irradiation. The apparent molecular mass in kDa is given above each trace.

**FIG. 6.** Size exclusion chromatography with Agp1-M15 (the N-terminal 504 amino acids of Agp1) apoprotein and chromophore adducts. a, apoprotein and BV adducts; b, 18EtBV adducts; c, 15Za adduct; d, 15Es adduct; e, 15Ea adduct. 

**FIG. 7.** Autophosphorylation of Agp1 apoprotein and chromophore adducts. The BV and 18EtBV adducts were used unirradiated (Pr) or after saturating red irradiation (Pfr). Following incubation in the presence of [γ-32P]ATP for 5 min, the samples were loaded to SDS-PAGE, blotted, and analyzed by phosphorimaging. The area around the 58-kDa Agp1 band is shown. The relative intensities (mean ± S.E. of four experiments) are given below the blot.

The 15Za and 15Es adducts revealed extraordinarily high activities, namely 380 and 300%, respectively. These data show that the syn chromophores impose a conformation on the protein that is different from the apoprotein, the Pr, and the Pfr form.

**DISCUSSION**

In the present study, four different synthetic BV chromophores in which rings C and D are stereocchemically locked were tested for their assembly with *Agrobacterium* phytochrome Agp1. Assays using desalting columns combined with UV-visible spectral measurements showed that all chromophores interact strongly with the Agp1 protein and form covalent adducts. A comparison of the absorption spectra (Figs. 2 and 3) gives detailed information about the conformation and configuration of the natural chromophore within the Agp1 chromophore pocket. The 15Za adduct is spectrally most similar to the Pr form of BV-Agp1 or 18EtBV-Agp1 and shows that in Pr the chromophore adopts a C15=C16 Z configuration and C14–C15 anti configuration. In plant phytochromes, the conformation of the double bond, which is stable during protein degradation, is known from NMR studies (9). An anti configuration of the single bond (as the E configuration of the double bond) has been proposed from vibrational spectroscopy (16), but a C14–C15 syn conformation has also been discussed (37).

The locked chromophore that forms an adduct most similar to Pfr is 15Ea. Therefore, in the Pfr form the chromophore has a C15=C16 E configuration and a C14–C15 anti conformation. The C15=C16 E configuration of plant phytochrome Pfr has also been shown by NMR (9). Based on vibrational spectroscopy, a C14–C15 syn conformation has often been proposed for the Pfr form (16). Recent density functional theory calculations predicted however that the Pfr chromophore is in the C14–C15 anti conformation (17), in accordance with our present findings. The assembly process of 15Ea is different from the other chromophores because it takes much longer until the absorbance changes are completed. The early adduct, which is formed just after mixing, has a rather low far-red absorbance and a λmax at 727 nm. The late adduct has a rather high absorbance and a λmax at 739 nm. The Pfr form of non-covalent Cph1-phycocyanobilin and Agp1-BV adducts (blocked or mutated covalent binding site) have a rather low absorbance and a blueshifted λmax as compared with the Pfr form of the covalent adducts (5, 6). We thus proposed that the transition from the early to the late Agp1–15Ea adduct reflects the formation of the covalent bond. This was indeed confirmed by the desalting column assay. These results are in line with a previous study in which Agp1 was assembled with (unlocked) 15E BV (38). In this study, a Pfr-like species with rather low absorbance and a blueshifted λmax was obtained after mixing. The 15E chromophore was not covalently bound to the protein; covalent bond formation was induced by dark reversion into 15Z-configured BV. Why is the covalent bond with 15Ea formed rather slowly as compared with the other chromophores? It could be that after the rapid incorporation of 15Ea into the Agp1 chromophore pocket, the distance between the Cys-20 sulfhydryl group and the ring A vinyl side chain of 15Ea is rather large and that the likelihood for covalent bond formation is thereby decreased. In this model, thermal conformational changes around the C5–C6 single bond is the most likely option. Although in vitro non-covalent phytochrome adducts can undergo photoconversion (5, 6), all known phytochrome proteins have a covalently bound chromophore in vivo. The present results show again that covalent bond formation is required for Pfr with optimized spectral properties.

What is the structural impact of the locked chromophores on the conformation of the Agp1 protein? To address this question, we have performed autophosphorylation and SEC measurements (Figs. 6 and 7). The phosphorylation activity of Pfr is lower than that of Pr. For SEC experiments, the truncated N-terminal chromophore module Agp1-M15 was used; the mobility of Agp1-M15 adducts is higher in the Pfr than in the Pr form. The apoprotein was also included in both assays, but differences between Pr and the apoprotein were rather small and the assays did not always allow us to distinguish between them. The 15Za adducts behave like Pr of the BV and 18EtBV adducts and the apoprotein. In limited proteolysis studies, which revealed differences between apo-Agp1 and the Pr form, the cleavage pattern of the 15Za adduct was indistinguishable from that of the Pr form (data not shown). For this reason, we assume that the protein conformation of the 15Za adduct is similar to or identical with the Pr form of Agp1.

The 15Ea adducts are very similar to the Pfr forms of the BV and 18EtBV adducts in SEC and autophosphorylation experiments. These data show that a Pfr-like form of phytochrome can be produced in darkness. Once the E isomer is present, the protein adopts its conformation in a proper manner. These findings open new ways for molecular phytochrome research.

*Agp1 Assembly with Locked Bilin Derivatives*
Locked 15Ea chromophores can be offered to plants that are defective in the synthesis of the phytochrome chromophore. The chromophores will most likely be taken up by the plants, as shown for other natural and synthetic chromophores (26, 39, 40). If the plant phytochrome assemblies with the locked 15Ea chromophore, a Pfr-like form will be produced irrespective of the light conditions. In this way, Pfr effects can be clearly distinguished from other light effects and can be well separated from effects of Pr/Pfr cycling.

The results with the 15Zs and 15Es chromophores show that the Agp1 protein is rather tolerant for bilins that have the “wrong” configuration. The absorption spectra of both adducts are more similar to those of heme adducts such as cytochromes than to phytochromes or other biliproteins. A comparable absorption spectrum with a blue absorption band at 421 nm was obtained with the C-terminal bilin binding fragment of cyanobacterial Cph2 (41). Both locked syn bilins might adopt a helical conformation within the Agp1 chromophore pocket as compared with the stretched conformation of other bilins. Another possibility could be that the conjugation system of tetrapyrrole chromophore is broken by a nucleophilic attack of an amino acid side chain to the C10 position. However, the addition reaction must be reversible, because the red absorption band appeared again upon SDS treatment of the holoprotein, as found during our tests for covalent binding. Both Agp1–15Zs and -15Es adducts behaved like the apoprotein (or Pr) on SEC but revealed an extraordinarily high autophosphorylation activity. Therefore, the protein can adopt at least four different distinguishable forms with respect to its kinase activity, the form of the apoprotein, Pr, Pfr, and that of the 15Zs or 15Es adduct. It is unclear how the C-terminal kinase of bacterial photolysomes is modulated by the N-terminal chromophore module. According to a recent model (20), the N and C termini of the dimeric photolychrome protein might be regarded as ends of a clothes peg (or a modified pair of scissors). In this model, Pr to Pfr photoconversion increases the distance between both C-terminal kinase subunits, because the interaction between both N-terminal chromophore modules is increased upon Pr/Pfr photoconversion. Incorporation of locked syn chromophores into Agp1 might result in an increase of the distance between both chromophore modules and in this way have a positive impact on kinase activity.

REFERENCES

1. Lamparter, T. (2004) FEBS Lett. 573, 1–5
2. Rüdiger, W., and Thümmler, F. (1994) in Photomorphogenesis in Plants (Kendrick, R. E., and Kronenberg, G. H. M., eds) pp. 51–69, 2nd Ed., Kluwer Academic Publishers, Dordrecht, The Netherlands
3. Hübschmann, T., Börner, T., Hartmann, E., and Lamparter, T. (2001) Eur. J. Biochem. 268, 2055–2063
4. Bhoo, S. H., Davis, S. J., Walker, J., Karniol, B., and Vierstra, R. D. (2001) Nature 414, 776–779
5. Lamparter, T., Michael, N., Mittmann, F., and Esteban, B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11628–11633
6. Borucki, B., Otto, H., Rottwinkel, G., Hughes, J., Heyn, M. P., and Lamparter, T. (2003) Biochemistry 42, 13684–13697
7. Lamparter, T., Carrascal, M., Michael, N., Martínez, E., Rottwinkel, G., and Abian, J. (2004) Biochemistry 43, 3659–3669
8. Lamparter, T., Michael, N., Caspari, O., Miyata, T., Shirai, K., and Inomata, K. (2003) J. Biol. Chem. 278, 33786–33792
9. Rüdiger, W., Thümmler, F., Cnael, E., and Schneider, S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6244–6248
10. Heyne, K., Herbst, J., Stelhik, D., Esteban, B., Lamparter, T., Hughes, J., and Diller, R. (2002) Biochemistry J. 82, 1094–1106
11. Holzwarth, A. R., Venuti, E., Braslavsky, S. E., and Schaffner, K. (1992) Biochem. Biophys. Acta 1140, 59–68
12. Zhang, C. F., Farrens, D. L., Björing, S. C., Song, P. S., and Kliger, D. S. (1992) J. Amer. Chem. Soc. 114, 4569–4580
13. Falk, H. (1989) The Chemistry of Linear Oligopyrroles and Bile Pigments, Springer Verlag Wien, New York
14. Andel, F. I., Murphy, J. T., Haas, J. A., McDowell, M. T., van der Hoef, I., Lagarias, J., Legewie, J., and Hartmann, M. A. (1999) Biochemistry 38, 2667–2676
15. Mizutani, Y., Tokutomi, S., and Kitagawa, T. (1994) Biochemistry 33, 153–158
16. Kneip, C., Hildebrandt, P., Schlamann, W., and Braslavsky, S. E. (1999) Biochemistry 16, 15185–15192
17. Moorthy, R. S., Murgida, D. H., von Stetten, D., Kneip, C., Mark, F., and Hildebrandt, P. (2004) J. Amer. Chem. Soc. 126, 16734–16735
18. Lagarias, J. C., and Mercurio, F. M. (1985) J. Biol. Chem. 260, 2415–2423
19. Chen, E., Lapko, V. N., Song, P. S., and Kliger, D. S. (1997) Biochemistry 36, 4903–4908
20. Esteban, B., Carrascal, M., Abian, J., and Lamparter, T. (2005) Biochemistry 44, 450–461
21. Yeh, K. C., Wu, S. H., Murphy, J. T., and Lagarius, J. C. (1997) Science 277, 1505–1508
22. Hübschmann, T., Jorissen, H. J., Börner, T., Gartner, W., and Tandeau de Marsac, N. (2001) Eur. J. Biochem. 268, 3383–3389
23. Yeh, K. C., and Lagarius, J. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13978–13981
24. Ryn, J. S., Kim, J. I., Kunkel, T., Kim, B. C., Cho, D. S., Hong, S. H., Kim, S. H., Fernandez, A. P., Kim, Y., Alonso, J. M., Ecker, J. R., Nagy, F., Lim, P. O., Song, P. S., Scharf, K., and Nam, H. G. (2005) Cell 129, 395–406
25. Ni, M., Tepperman, J. M., and Quid, P. H. (1999) Nature 400, 781–784
26. Hanawa, H., Shinomura, T., Inomata, K., Kikuchi, T., Kinoshita, H., Wada, K., and Furuya, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4725–4729
27. Hanawa, H., Inomata, K., Kinoshita, H., Kikuchi, T., Jayasundera, K. P., Sawamoto, D., Ohtta, A., Uchida, K., Wada, K., and Furuya, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3612–3617
28. Lindner, I., Knapp, B., Braslavsky, S. E., Gartner, W., and Schaffner, K. (1998) Angew. Chem. Int. Ed. Engl. 37, 1843–1846
29. Kikuchi, T., Hatakeyama, M., Inomata, K., Wada, K., and Furuya, M. (1998) Chem. Lett. 1001–1002
30. Kikuchi, T., Kinoshita, H., and Inomata, K. (1999) Synlett, 901–904
31. Sawamoto, D., and Inomata, K. (2001) Chem. Lett. 588–589
32. Takeda, S., Jayasundera, K. P., Kikuchi, T., Kinoshita, H., and Inomata, K. (2001) Chem. Lett. 590–591
33. Kinoshita, H., Hammam, M. A. S., and Inomata, K. (2005) Chem. Lett. 34, in press
34. Hammam, M. A. S., Murata, Y., Kinoshita, H., and Inomata, K. (2004) Chem. Lett. 123, 1258–1259
35. Lamparter, T., Esteban, B., and Hughes, J. (2001) Eur. J. Biochem. 268, 4720–4730
36. Otto, H., Lamparter, T., Borucki, B., Hughes, J., and Heyn, M. P. (2003) Biochemistry 42, 5885–5895
37. Tokutomi, S., Sugimoto, T., and Mimuro, M. (1992) Photocomm. Photobiol. 56, 545–552
38. Lamparter, T., and Michael, N. (2005) Biochemistry, in press
39. Parks, B. M., and Quail, P. H. (1991) Plant Cell 3, 1177–1186
40. Lamparter, T., Eech, H., Cove, D., Hughes, J., and Hartmann, E. (1996) Plant Cell Environ. 19, 560–569
41. Wu, S. H., and Lagarius, J. C. (2000) Biochemistry 39, 13487–13495

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