Blue Light Induces Radical Formation and Autophosphorylation in the Light-sensitive Domain of Chlamydomonas Cryptochrome

Blue light governs many responses of organisms to environmental conditions. Cryptochromes have been shown to act as sensory blue light photoreceptors in plants and animals, with their action being diverse as their origin (1). From sequence analysis, cryptochromes have been divided into three subgroups: animal, plant, and DASH cryptochromes (2). Animal cryptochrome synchronizes the circadian clock of Drosophila to the 24-h rhythm (3). In mammals, cryptochrome has been suggested to be involved in circadian entrainment (4), and it functions independent of light as a main component of the biological clock. Arabidopsis cryptochromes 1 and 2 (AtCRY1(2) and AtCRY2) mediate de-etiolation responses, entrain the circadian clock, trigger programmed cell death induced by singlet oxygen, and regulate flowering time, stomatal opening, and production of anthocyanin (5–8). DASH cryptochromes are mostly found in bacteria but also in Neurospora crassa, aquatic vertebrates (9), and Arabidopsis (AtCRY3) (10). Their putative role as sensory receptors has recently been challenged by showing that they act as repair enzymes for single-stranded DNA (11).

The 500 N-terminal amino acids constitute the photolyase homology region (PHR) with reference to the DNA repairing enzyme. In this domain all cryptochromes characterized so far carry flavin adenine dinucleotide (FAD) as light-sensitive chromophore (2, 12, 13). Similarity in structure to the photolyases suggests that 5,10-methenyltetrahydrofolate (MTHF) might be bound as a second (antenna) chromophore. However, only a few preparations of plant cryptochromes contain MTHF (14), which might be explained by a reduced affinity to the altered binding pocket (15). Plant cryptochromes are characterized by a long C-terminal extension that varies considerably in size and sequence. Fusion of the extension with β-glucuronidase leads to a constitutively active phenotype for AtCRY1 and AtCRY2, which implies a role of this domain for signal transduction (16).

All spectroscopic data on the blue light response of plant cryptochromes have been reported on Arabidopsis cryptochromes. Interpretation of the data has been facilitated by the availability of a crystal structure of the AtCRY1-PHR domain (15). In AtCRY1, absorption of blue light induces formation of a neutral flavin radical, which decays on a millisecond time scale (17). Results from fluorescence and electron paramagnetic resonance experiments on whole insect cells containing AtCRY1 indicate that the radical is the active state of the receptor in vivo (18). This radical shows a unique spectroscopic signature in the visible and infrared spectral region that distinguishes it from that of the homologous photolyases (19). Results from transient absorption spectroscopy and site-directed mutagenesis showed that a conserved tryptophan triad and a tyrosine residue are included from infrared difference spectroscopy that a crucial step of the photolyase homologues in the visible and infrared spectral region that distinguishes it from that of the homologous photolyases (19). Results from transient absorption spectroscopy and site-directed mutagenesis showed that a conserved tryptophan triad and a tyrosine residue are included from infrared difference spectroscopy that a crucial step of the photolyase homologues.
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by a nearby aspartic acid (19). Further downstream in the signal cascade, blue light induces autophosphorylation of AtCRY1 in vitro and in vivo (21, 22) and was proposed to change the interaction with the ubiquitin-protein isopeptide ligase COP1 (23, 24).

A single putative member of the plant cryptochrome family has been identified in the unicellular green alga *Chlamydomonas reinhardtii* (*Chlamydomonas* photolysase homologue 1 CPH1). As a possible evolutionary precursor of plants (25), the CPH1 gene product is closely related to AtCRY1 with 49% identity in the first 500 amino acids (26). CPH1 contains by far the longest C-terminal extension (507 amino acids) compared with all known cryptochromes (7). Initial expression of full-length CPH1 yielded a 110-kDa product from *E. coli* but 126- and 143-kDa proteins from *Chlamydomonas* as revealed by Western blot analysis (27). Size discrepancies were hypothesized to be due to posttranslational modifications. In vivo, CPH1 undergoes light-induced proteolysis (27), but the physiological function of CPH1 remains elusive. It has been suggested that CPH1 is involved in regulation of gene transcription and/or circadian phototaxis rhythmicity (27).

Here, we report on a high yield production of the light-sensitive domain of *C. reinhardtii* cryptochrome in *E. coli*. Blue light induces formation of the radical state similar to full-length *Arabidopsis* CRY1. We demonstrate that ATP binding strongly influences the properties of the domain and enables light-induced auto-phosphorylation in the absence of the C-terminal domain.

**EXPERIMENTAL PROCEDURES**

DNA manipulation, cloning, and transformation of *E. coli* were done by standard techniques (28). Oligonucleotides were purchased from MWG Biotech or Invitrogen. Restriction enzymes were from New England Biolabs, Invitrogen, or Fermentas. Sequencing of DNA was performed by dideoxy sequencing using Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Biosciences) and the LI-COR 4200 Gene ReadIR (MWG Biotech). 5, 10-Methylene-tetrahydrofolate (MTHF) was a kind gift from Dr. R. Moser (NYT Biotech). 5, 10-Methenyltetrahydrofolate (MTHF) was a kind gift from Dr. R. Moser (Merck).

**Construction of Expression Vector**—The sequence encoding the N-terminal 504 amino acids of CPH1 from *C. reinhardtii* (NCBI accession code L07561) was amplified by PCR. A coding region for six histidines was added downstream to the start codon. The PCR product was ligated into pCR 2.1 (Invitrogen). *E. coli* strain TOP10 (Invitrogen) containing the expression construct was grown in lysogeny broth medium supplemented with 200 mg/liters ampicillin and 200 μM riboflavin at 37 °C in the dark. When the optical density at 600 nm reached 0.5, the temperature was lowered to 18 °C. At an optical density of 0.7, isopropyl-β-D-1-thiogalactoside was added to a final concentration of 10 μM. After 20 h, the cells were harvested by centrifugation (6,000 × g, 20 min, 4 °C). The cell pellet was resuspended in 0.2 M Tris, pH 8, 0.3 M NaCl, 20% glycerol, 1 tablet of protease inhibitor mixture (Complete EDTA-free; Roche Applied Science) and DNase. The cells were disintegrated using French Press (3 × 1000 psi). The lysate was centrifuged (100,000 × g, 1 h, 4 °C) and loaded onto a *Strep*-Tactin-Sepharose column (IBA). After washing with 0.2 M Tris, pH 8, 0.3 M NaCl, 20% glycerol, the protein was eluted in the same buffer supplied with 2.5 mM D-dithiothreitol. The isolated holoprotein is stable for several days at 4 °C.

**Protein Analysis**—The construct was detected on Western blot (28) using an antibody against the *Strep* tag (IBA) and the *Strep* tag horseradish peroxidase detection kit (IBA) or an antibody against the polyhistidine tag (Sigma) and Super Signal West Pico Chemiluminescent substrate (Pierce). MALDI-TOF analysis was performed after in-gel digestion with trypsin as described previously (29). For determination of the protein concentration, the protein was diluted in 6 M guanidinium hydrochloride. The extinction coefficient of the apoprotein at 280 nm was calculated with ProtParam (www.expasy.org). A chromatophore content of up to 70% was estimated by the ratio A280/A550 using an extinction coefficient for FAD in solution of 11300 M⁻¹ cm⁻¹ at 450 nm (30).

**Folate Assay**—In a modified fluorescence assay for folate (31), CPH1-PHR was treated as follows. 0.2 μl of concentrated HCl was added to 25 μl of sample and heated for 3 min at 99 °C. The precipitate was removed by centrifugation and the supernatant diluted to 200 μl. In the next step, sodium hydroxide (10 M) was added until pH 12–13 was reached. Finally, the sample was oxidized by 10 μl of a KI/I₂ (20%/10%) solution and overnight incubation. After each step, fluorescence spectra were recorded with a Shimadzu RF 1501 spectrophotofluorometer.

**Photoreduction and Kinetics of Recovery**—UV-visible absorption spectra were recorded with a Uvikon 943 (Kontron Instruments) or a Shimadzu 2401 PC spectrophotometer. The buffer was exchanged to 0.2 M Tris, pH 8, 0.3 M NaCl, 20% glycerol prior to photoreduction, and the sample was kept at 10 °C.

**Phosphorylation Assay**—The recombinant protein isolated from *E. coli* was dephosphorylated in the dark. Phosphatase treatment was performed at 4 °C for 2.5 h in a buffer containing 36 μM CPH1-PHR, 40 mM Tris, 40 mM NaCl, 10 mM MgCl₂, 8% glycerol, 5 mM KCl, 10 μM ZnCl₂, and 7.5 units of calf intestine alkaline phosphatase (CIAP) (Fermentas). After the treatment, 10 μl of 1.7 mM Na₃VO₄ for inhibition of the CIAP (32), 10 μl of 100 mM MgCl₂, and 10 μl of 33 mM ATP were added to 70 μl of the sample solution. All samples were kept at 25 °C and shaken.
in a thermodixer at 500–1000 rpm (Eppendorf). To prevent heating of the sample by irradiation, a 457-nm light-emitting diode was used (20 nm full width at half maximum; Luxeon Star Lumileds). It provided 77 microwatt/cm² of blue light at the sample. Dark samples were covered with aluminum foil. Reactions were stopped by the addition of SDS-PAGE sample buffer (28) containing 0.4 M dithiothreitol, 8 M urea, 10% sodium dodecyl sulfate, and by boiling for 15 min at 95 °C. These harsh denaturing conditions were applied to ensure complete dissociation of ATP from the protein. After performing electrophoresis with a 12% polyacrylamide gel, phosphorylated protein was stained with the Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes) (33) and detected with 302 nm UV light (Gel Doc; Bio-Rad). Total protein was made visible afterward by Coomassie staining. Signal strength in the phospho-stain was analyzed in reference to the background and normalized to the protein amount from the Coomassie stain by ImageJ software (NIH Image, rsb.info.nih.gov/nih-image/index.html).

RESULTS

CPH1 from the unicellular green alga C. reinhardtii is a member of the plant cryptochrome family according to sequence analysis. Its function and properties are unknown; therefore we set out to study the biochemical and spectroscopic characteristics of the CPH1 light-sensitive domain.

Construction of the Expression Vector—The 504-amino acid boundary of the N-terminal photolyase-like domain was determined using sequence alignment and available structural data of AtCRY1 and E. coli DNA photolyase in conjunction with secondary structure prediction and hydropathy analysis. Sequencing of the cDNA revealed a deviation in the construct as compared with the published sequence. Codon 314 was found to be cgc instead of ggc, which would result in a mutation from Gly to Arg. In a control experiment, genomic DNA from a C. reinhardtii extract (P. Hegemann, Berlin, Germany) was used as a PCR template to amplify a part of the CPH1 sequence containing codon 314. The PCR product was cloned and analyzed by sequencing, confirming the deviation from the published sequence. This implies that amino acid at position 314 of CPH1 is indeed arginine.

Protein Expression and Purification—Using standard expression conditions for E. coli (37 °C, 3 h, 1 mM isopropyl-1-thio-β-D-galactopyranoside), the protein was obtained in insoluble form as inclusion bodies. It was solubilized with 1% N-laurolysarcosine and further purified. However, attempts to reconstitute the protein with FAD as a chromophore were not successful. Modification of the protocol to very mild expression conditions (see “Experimental Procedures”) yielded up to 20 mg of a soluble, yellow-colored protein/liter of induced cell culture. SDS-PAGE showed a band at ~60 kDa with a purity of >90% (Fig. 1, lanes A and C). The experimentally determined molecular mass corresponds to the theoretical mass of the apoprotein of 59.13 kDa. The recombinant protein was detected using antibodies against the N-terminal polyhistidine tag and the C-terminal Strep tag (Fig. 1, lanes B and D). The identity of the CPH1-PHR construct was further confirmed by MALDI-TOF mass spectrometry. The protein was subjected to tryptic digestion. Matched peptides covered 21% (109/520 amino acids) of the construct (data not shown).

Identification of the Chromophores—A yellow protein was obtained after affinity chromatography. The UV-visible spectrum showed a fine-structured (“three-finger”) absorption band in the 400–500-nm region and a broad absorption in the 300–400-nm region with maxima at 368 and 449 nm (Fig. 2A). These are typical features of an oxidized flavoprotein. As heterologously expressed flavoproteins may incorporate different flavins such as riboflavin, FMN, or FAD (34), the chemical nature of the cofactor was determined by fluorescence spectroscopy. CPH1-PHR was heat denatured, and the precipitate was removed by centrifugation. Before and after denaturation, fluorescence excitation spectra with emission at 528 nm and emission spectra with excitation at 450 nm were recorded (Fig. 2B). Maxima were detected at 364 and 448 nm in the excitation spectrum and at 529 nm in the emission. Upon acidification with HCl the fluorescence signal of the free chromophore rose approximately by 4-fold (Fig. 2B). This rise is a peculiarity of FAD caused by a loss of interaction between the adenine and flavin ring systems in acidic solution (35). In a more quantitative approach, the chromophore composition was further analyzed by thin layer chromatography (TLC). Using authentic FAD as a standard, the isolated chromophore showed a signal at the same height without contributions from other flavin species (supplemental Fig. S1). The results from UV-visible spectroscopy, fluorescence spectroscopy, and TLC analysis identified oxidized FAD as the exclusive flavin chromophore of CPH1.

It has been reported that AtCRY1 contains MTHF as a second chromophore (14), which is absent in most preparations (15, 19). As CPH1 is highly homologous to AtCRY1, CPH1 might also bind MTHF. The chromophore was separated from the CPH1-PHR apoprotein and investigated with a fluorescence assay for folates (31) (Fig. 3). Upon excitation at 350 nm, the fluorescence spectrum showed a prominent flavin emission at 528 nm. Additionally, a weak band at 441 nm was present, which is indicative for folates. The supernatant was basified by sodium hydroxide and finally oxidized by adding KI/I₂. The decay of the 441 nm fluorescence after addition of base and...
the subsequent strong increase by oxidation are strong indications for the presence of a folate as a second chromophore in CPH1. Bases catalyze the hydrolysis of the methenyl bridge of MTHF to the nonfluorescent 10-formyltetrahydrofolate (31). The oxidizing agent then produces fluorescent products. It should be pointed out, however, that the amount of detected folate was minute and it was only observed in a few preparations.

The Response of the Chromophore to Blue Light Illumination—
The effect of blue light on CPH1-PHR was followed by UV-visible spectroscopy under aerobic conditions without external electron donor. Illumination at \( \lambda = 445 \text{ nm} \) caused a decrease of the absorption band at 449 nm, which shows the disappearance of oxidized flavin (Fig. 4A). Simultaneously, formation of a broad absorption band between 500 and 600 nm with maxima at 540 and 580 nm was observed. This absorption is indicative for a blue neutral flavoprotein radical (36). Two isosbestic points at 354 nm was lost after illumination for more than 1 min, indicating partial formation of the fully reduced state of CPH1-PHR. Even under anaerobic conditions, prolonged illumination (> 1 min) was necessary to initiate the production of the fully reduced state (data not shown). It has been reported that AtCRY1 binds ATP (21) to the PHR domain (15). Addition of ATP to CPH1-PHR led to a strong enhancement in light-induced radical formation (Fig. 4B). Isosbestic points did not change even after 300 s of illumination. The presence of AMP-PNP, a nonhydrolyzable analog of ATP, resulted in a light response identical to that with ATP (Fig. 4C). This result shows that the effect is due to ATP binding to the protein and not related to protein phosphorylation. The enhancement in radical formation is specific for ATP, because it was not inducible by addition of GTP (data not shown).

The decay of the CPH1-PHR radical in the dark was investigated in a separate series of experiments. The sample was illuminated for 10 s with 457 nm blue light, and the recovery of the oxidized state was monitored through the absorption at 450 nm (Fig. 5A). The kinetic measurements were performed aerobically in the presence of the exogenous electron donor 2-mercaptoethanol. Initially, time traces were not fully reproducible and the sample did not fully recover back to the oxidized state. These problems were attributed to a deprivation of oxygen in the sample during illumination. Treatment with oxygen prior to illumination led to recovery kinetics of the oxidized state, which follow a monoexponential shape with a time constant of \( t = 200 \text{ s} \). The UV-visible spectrum obtained after recovery was identical to the one recorded before illumination (Fig. 5A, inset), implying that the light-induced reaction was fully reversible. Addition of ATP to the sample strongly impeded the radical decay (Fig. 5B). Only a slight recovery of the 450 nm absorption of the oxidized flavin was observed within the experimental time window of 30 min, which is illustrated by comparison with a theoretical curve with a time constant of 200 s (Fig. 5B, dashed line). Similarly, the decay kinetics was
strongly slowed down under anaerobic conditions and was not interpretable by a simple kinetic scheme (data not shown). These findings indicate that ATP and oxygen concentration are major determinants of the decay kinetics of the CPH1-PHR radical.

The Response of the Domain to Blue Light: a Phosphorylation Assay—We hypothesized that CPH1 undergoes a blue light-dependent autophosphorylation as has been claimed for AtCRY1 (21, 22). The phosphorylation assay demonstrated that the CPH1-PHR is isolated from *E. coli* in a strongly phosphorylated state (Fig. 6A, lane 1). To determine blue light-induced phosphorylation, it was necessary to initially dephosphorylate the protein. This was achieved by incubation with CIAP at 4 °C in the dark, by which CPH1-PHR was dephosphorylated to a basal level (Fig. 6A, lane 2). In the next step, the phosphatase was inhibited by the addition of Na3VO4. ATP and MgCl2 were added as substrate and cosubstrate in the phosphorylation reaction. Illumination with blue light for 10, 20, and 30 min induced a significant increase of the signal at 60 kDa on the phospho-stain gel as compared with the dark-treated samples incubated for the same time (Fig. 6A). Equal loading of the protein was visualized by Coomassie staining (Fig. 6B). It was concluded that the illuminated samples are stronger phosphorylated than the dark-treated samples, which implies that
CPH1-PhR undergoes a blue light-dependent autophosphorylation. Incubation at 25 °C in the dark did not increase the phosphorylation level within the accuracy of the detection. When illuminated, the sample tended to aggregate and therefore was detected at higher masses and in the stacking gel, especially at longer illumination times (supplemental Fig. S2). These aggregates are highly phosphorylated and could not be fully separated on the gel. An additional band was detected between the 60-kDa band of CPH1-PhR and the 66-kDa band of the standard (Fig. 6). This band is assigned to CIAP, which is active as a dimer of two 65-kDa subunits (37). Faint bands of the CIAP are observed in the Coomassie-stained gel that give strong signals on the phospho-stain. These signals are attributed to a fraction of strongly phosphorylated CIAP from the catalyzed hydrolysis of ATP, despite the inhibition by Na3VO4. This interpretation was supported by a control experiment without CPH1-PhR (supplemental Fig. S3). Even before the addition of ATP, CIAP was detected as a weak signal on the phospho-stain gel (Fig. 6, lane 2). In this case, the substrate for CIAP might be phosphorylated CPH1-PhR.

**DISCUSSION**

We report on the successful heterologous overexpression of a plant cryptochrome in *E. coli*. Until now, only *Arabidopsis* CRY1-PhR has been obtained from *E. coli* as a fusion to maltose-binding protein (14, 15), whereas full-length plant cryptochromes remain inaccessible from prokaryotic expression systems. The N-terminal photolyase homology region of *C. reinhardtii* CPH1 forms a neutral flavoprotein radical in response to blue light concomitant with an increase in phosphorylation (Fig. 7). The recovery of oxidized FAD proceeds in the order of minutes in the dark and is strongly delayed by the presence of ATP or the absence of oxygen.

**CPH1 Is a Member of the Plant Cryptochrome Family**—Phylogenetic analysis groups *Chlamydomonas* CPH1 into the family of plant cryptochromes, in which CPH1 might play an ancestral role (25). This assignment is supported by the fact that only one plant cryptochrome is found in the algal genome, whereas other cryptogams such as *Physcomitrella* or *Adiantum* have two and five genes, respectively (38). The PHR domain of CPH1 shows a 49% identity to AtCRY1 (26) and 31% to *E. coli* DNA photolase. Its physical and chemical properties identify CPH1 as a plant cryptochrome: The flavin chromophore is found characteristic for plant cryptochromes, as it has been shown for AtCRY1 (13). In contrast, photolyases and members of the Cry-DASH family contain FAD in its oxidized, radical, or fully reduced form depending on enzyme and preparation procedure (39–41). Illumination of CPH1-PhR with blue light leads to formation of a neutral flavoprotein radical indicated by a broad absorption band with maxima at 540 and 580 nm. The maximum at 580 nm is strongly blue-shifted by more than 40 nm in comparison to the spectra of the radicals in *E. coli* photolase (42) or DASH cryptochrome AtCRY3 (39). This shift is characteristic for plant cryptochromes, as it has been shown for AtCRY1 (19), and is even more pronounced in CPH1. In comparison with other plant cryptochromes, the C-terminal extension does not contain the conserved DQXVP-acidic-STAES motif (7, 43) nor does it show any known motifs besides a high amount of Gly (25%) and Ala (16%) residues.

**The Chromophores in CPH1**—The recombinant N-terminal domain of CPH1 purified from *E. coli* bears FAD in its oxidized state. The presence of riboflavin or FMN in the binding pocket of CPH1 is excluded on the basis of TLC analysis (supplemental Fig. S1). This points to an important contribution of the ade-
nine moiety of FAD to binding of the chromophore in cryptochromes. It has been shown for the homologous DNA photolyase from E. coli that riboflavin and FMN fail to bind to the apoprotein (44). These findings are in contrast to other blue light receptor domains such as the light-, oxygen-, and voltage-sensitive (LOV) domains of phototropin, where reconstitution with flavin analogs has been achieved (45), or the sensors of blue light using FAD (BLUF) domain, where expression conditions determine the chromophore composition (34).

Low amounts of a folate were detected by fluorescence analysis in a few preparations of CPH1-PHR (Fig. 3). It has been reported that AtCRY1 contains MTHF as a second chromophore (14). Preservation of functional MTHF during the preparation procedure is complicated by the fact that it decomposes once released from the apoprotein at pH 8 (31). Gel filtration experiments on CPH1-PHR showed that folate is predominantly present in aggregates (data not shown). We suggest that multimerization facilitates retaining the MTHF chromophore in CPH1. However, it is not clear yet whether plant cryptochromes bear functional MTHF at all. The high extinction coefficient of oxidized flavin might allow for blue light sensing without the necessity for MTHF as an antenna chromophore.

Radical Formation in CPH1-PHR upon Blue Light Illumination—The response to blue light differs among the cryptochromes, making it necessary to discuss the subgroups individually. In the plant cryptochrome AtCRY1, blue light induces a transition from the oxidized state of flavin to the neutral radical FADH* (13, 17), whereas in the animal cryptochrome dCRY from Drosophila an anionic radical FAD* is formed (46). In the DASH cryptochrome AtCRY3, the reaction proceeds via the neutral radical to the fully reduced state of flavin FADH2 (47). CPH1-PHR responded very similarly to illumination as AtCRY1. During the illumination process two species were present exclusively in the sample, i.e. the oxidized and the neutral radical state of the flavoprotein (Fig. 4). Only prolonged, intense illumination produced the fully reduced state of CPH1-PHR.

Radical formation under anaerobic conditions and with external electron donors is a common feature of flavoproteins (40) and does not allow drawing any meaningful conclusion on physiological processes. In contrast, the present experiments on CPH1-PHR were performed aerobically without any exogenous electron donor (Fig. 4). Formation of a stable radical was observed despite the fact that the experimental conditions were favoring the oxidized state of the flavin. Therefore, we propose in analogy to Arabidopsis cryptochromes that the oxidized state is the native dark state. We consider the flavoprotein radical as being physiologically active in Chlamydomonas as has been claimed for AtCRY1 and AtCRY2 (18–20, 48). The critical residues that have been proposed to be involved in signal transduction in AtCRY1, i.e. Trp-324 (17), Trp-400 (20), and Asp-396 (19), are all conserved in CPH1. For cry-DASH proteins, such as CRY1 from Vibrio cholerae, the fully reduced state has been proposed to represent the dark form (40). A clear differentiation between plant and DASH cryptochrome is necessary, considering the recent finding that cry-DASH members act as photolyases for single-stranded DNA (11).

The decay of the radical in the dark proceeds on the order of minutes. In a solution saturated with oxygen and in the presence of 2-mercaptoethanol, the time constant was determined to be ~200 s. This is comparable with the dark recovery of AtCRY2 with a time constant of 360 s (t1/2 = 250 s) (48). For AtCRY1, Giovani et al. (17) demonstrated that the neutral flavin radical decayed already in milliseconds, but did not resolve a contribution with a half live of >100 ms. Time-resolved measurements in the subsecond time regimen need to be conducted on CPH1 to resolve this issue. The concentration of oxygen is a limiting factor for the decay of the radical in CPH1-PHR. If the sample is depleted with oxygen during illumination, the recovery kinetics of the oxidized state is slowed down, because oxygen has to diffuse from the atmosphere into the sample solution and further into the chromophore binding pocket.

Effect of ATP Binding to the Blue Light Receptor Domain—The crystal structure is available of the AtCRY1-PHR domain with bound AMP-PNP (15). The nucleotide occupies the site of substrate binding postulated for DNA photolyases. The analysis did not reveal any structural changes upon binding. The effect of ATP on the cryptochrome light reaction has not been studied before. The observed strong enhancement in light-induced radical formation of CPH1-PHR is primarily an effect of a slower dark recovery and not of an increase in quantum yield of formation of the radical. A small stabilizing effect of the substrate on the enzyme has been reported for DNA photolyase (49). Upon binding of the cyclobutane pyrimidine dimer, the reduced state of the enzyme is more resistant to air oxidation due to a rise in reduction potential by 65 mV. In contrast, ATP binding leads to a strong stabilization of the radical state of CPH1 with reference to both oxidized and reduced states. This can be deduced from the much slower recovery of oxidized flavin (Fig. 5B) and the absence of any fully reduced state in the sequence of light-induced absorption spectra (Fig. 4B), respectively. Together with the observed autophosphorylation (see below), these findings indicate that nucleotide binding is of functional significance for plant cryptochromes and not merely caused by structural similarity in the binding pocket to DNA photolyase. ATP is abundant in cells and shows high affinity to PHR domains with a dissociation constant (Kd) of 20 μM (as determined for AtCRY1) (21). As a consequence, experiments on plant cryptochromes need to be conducted in the presence of ATP or ATP analogs to draw meaningful conclusions with regard to the in vivo situation.

Light-dependent Autophosphorylation of CPH1-PHR—We have shown that CPH1-PHR performs blue light-dependent autophosphorylation under in vitro conditions (Fig. 6). This reaction is surprising because the native C-terminal extension is missing in the construct. It is not clear whether the observed autophosphorylation of the PHR domain reflects a specific response of the full-length protein in vivo. However, the level of phosphorylation detected speaks against an unspecific overphosphorylation as found, for example, in rhodopsin in vitro (50). Autophosphorylation has already been demonstrated for full-length AtCRY1 and AtCRY2 in vitro and in vivo (21, 22, 51) despite the fact that they do not contain sequence similarities to any known kinase. It is closely associated with the function or regulation of the receptors (22, 51). The PHR domain is suffi-
cient for this response in AtCRY1. Our results imply that the C-terminal extension is dispensable for the phosphorylation response and that the ability to undergo light-induced auto-phosphorylation is a characteristic feature of plant cryptochromes. The lack of homology of the C-terminal extensions between algal and flowering plant cryptochromes indicates that a conserved phosphorylation response might indeed be limited to the PHR domain.

A recent study has challenged the influence of light to the autophosphorylation process in AtCRY1 (52). The authors determined only a minor influence of light. In comparison to our study on CPH1-PHR, illumination conditions were applied with 31 microwatt/cm² as compared with 77 microwatt/cm² intensity and 360 nm light instead of 445 nm. Particularly the shorter wavelength might strongly reduce the reaction efficiency via enhanced internal conversion as was found in the flavin-containing light-, oxygen-, and voltage-sensitive domains of Chlamydomonas phototropin (53). Furthermore, detection of a phosphorylation increase by radiostaining instead of using a fluorescent dye might be limited if proteins are already phosphorylated to significant levels prior to the study (33).

From the crystal structure analysis of the AtCRY1-PHR domain with bound ATP analog it was suggested that the nature of the autophosphorylation reaction is intermolecular, implying a dimerization reaction with a second AtCRY1 molecule (15). Dimerization might be necessary to shorten the distance of the γ-phosphate of ATP to a putative acceptor, which is in the monomer ~11 Å. In support of this proposal, we observed a light-induced multimerization of CPH1-PHR (supplemental Fig. S2). It is tempting to speculate that the blue light-induced autophosphorylation and aggregation of CPH1-PHR we observed in vitro are correlated with the physiological function in Chlamydomonas. CPH1 undergoes light-induced proteolysis in vivo, which is eliminated by the addition of kinase inhibitors (27). From this observation it was concluded that phosphorylation of CPH1 might be required for light-induced degradation. The photosensory function of CPH1 in Chlamydomonas is yet unknown. A study using an RNA interference approach is in preparation.

CONCLUSIONS

More than 11 years have passed since the first absorption spectrum of a plant cryptochrome was published (13). Since then, the understanding of the molecular processes after blue light illumination has only gradually improved. This is partly due to demanding sample preparation. The Chlamydomonas CPH1-PHR domain is now available in sufficient yield and purity from heterologous expression for investigation with biochemical techniques such as ultrafast spectroscopy or Fourier transform infrared spectroscopy. This approach has been very fruitful for the blue light receptor phototropin, where many details of the reaction mechanism have been elucidated within a few years. We have demonstrated that the C-terminal extension of cryptochromes is dispensable for light-induced autophosphorylation. Therefore, our eukaryotic model domain with its high homology to AtCRY1 is a good candidate to study two fundamental light-induced processes of full-length plant cryptochromes: radical formation and autophosphorylation.

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