Hyperactivity of the Arabidopsis cryptochrome (cry1) L407F mutant is caused by a structural alteration close to the cry1 ATP-binding site

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Plant cryptochromes (cry) act as UV-A/blue light receptors. The prototype, Arabidopsis thaliana cry1, regulates several light responses during the life cycle, including de-etiolation, and is also involved in regulating flowering time. The cry1 photocycle is initiated by light absorption by its FAD chromophore, which is most likely fully oxidized (FADox) in the dark state and photoreduced to the neutral flavin semiquinone (FADH+) in its lit state. Cryptochromes lack the DNA-repair activity of the closely related DNA photolases, but they retain the ability to bind nucleotides such as ATP. The previously characterized L407F mutant allele of Arabidopsis cry1 is biologically hyperactive and seems to mimic the ATP-bound state of cry1, but the reason for this phenotypic change is unclear. Here, we show that cry1/L407F can still bind ATP; has less pronounced photoreduction and formation of FADH+ than wild-type cry1, and has a dark reversion rate 1.7 times lower than that of the wild type. The hyperactivity of cry1/L407F is not related to a higher FADH+ occupancy of the photoreceptor but is caused by a structural alteration close to the ATP-binding site. Moreover, we show that ATP binds to cry1 in both the dark and the lit states. This binding was not affected by cry1’s C-terminal extension, which is important for signal transduction. Finally, we show that a recently discovered chemical inhibitor of cry1, 3-bromo-7-nitroindazole, competes for ATP binding and thereby diminishes FADH+ formation, which demonstrates that both processes are important for cry1 function.

Cryptochromes and DNA photolases constitute a superfamily of flavoproteins (cryptochrome/photolyase family), with members present in all kingdoms of life. DNA photolases repair the two main UV lesions in DNA, cyclobutane pyrimidine dimers (CPD)2 and pyrimidine-pyrimidone (6–4) photoproducts, whereas cryptochromes are UV-A/blue light photoreceptors, essential components of the circadian clock, or putative magnetic field sensors (1–6). The superfamily includes at least six subfamilies (7). One of these subfamilies consists of plant cryptochromes, of which Arabidopsis thaliana cry1 (Atcry1) was the first member identified (8). cry1 together with the other plant cryptochrome (Atcry2) regulates many of the responses of A. thaliana to blue light, in particular deetiolation and photoperiodic flowering (6) but also plant growth under low blue light conditions in the shade (9).

Cryptochromes and DNA photolases possess a similar protein fold that accommodates flavin adenine dinucleotide (FAD) in the α-helical domain of the photosensory domain, also known as the photolyase-homology region (PHR), but the redox state of the flavin differs. In photolyses, the flavin is in its fully reduced and photoexcited state FADH−, which donates an electron to the DNA lesion to start the catalytic cycle (1). The two reduced states of FAD, i.e. the radical and the fully reduced state, are achieved at least in vitro by photoreduction of FAD. In this process, FAD receives an electron from a nearby tryptophan residue. The electron gap is filled by two other tryptophan residues that form a Trp triad with the Trp electron donor; the most distal Trp at the protein surface is finally reduced by an external reductant (3, 10, 11). By contrast, it is believed that the signaling state of photoactive cryptochromes contains a flavin radical, either the anionic radical or the neutral semiquinone, and the dark state contains FADox (12–17). Other models of the cryptochrome photocycle suppose that the ground (dark) state is represented by the anionic radical and the lit state is a somehow photoexcited form of the anionic radical FAD− (4, 10). This would mean that the Trp triad is not required in vivo, at least not for electron transfer, as concluded from the analysis of Trp-triad mutants of Arabidopsis cry1 and cry2. Some of these mutants still respond to light, whereas other Trp-triad mutants show a constitutive photomorphogenic (cop) phenotype (18, 19). There is, however, some doubt that the lifetime of a photoexcited anionic radical would be sufficient to allow a confor-

2 The abbreviations used are CPD, cyclobutane pyrimidine dimer; pCry, plant-type cryptochrome; PHR, photolyase–homology region; AMP-PNP, adenylyl-imidodiphosphate; CCE, cryptochrome C-terminal extension; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PDB, Protein Data Bank; ITC, isothermal titration calorimetry; 387N, 3-bromo-7-nitroindazole.
mational change in the protein moiety and thus signal transduction (20). Plant cryptochromes accumulate the neutral FAD semiquinone \textit{in vitro} during illumination (12, 13, 18–21), and this process depends on an intact Trp triad (18, 19, 22). FADH$^+$ can be obtained only if both electron transfer and proton transfer occur. In \textit{Arabidopsis} cry1, Asp-396 serves as the proton donor to the N5 atom of the isoalloxazine ring of FAD (23).

Photolyases and cryptochromes differ in another aspect, namely the C-terminal extension (CCE) of cryptochromes. Such extensions are also found in DASH-type cryptochromes, which have residual activity for repairing CPDs in single-stranded DNA (24, 25) and, in several fungi, also a photoreceptor function (26–29). The CCE in cry1 and cry2 can mediate signaling (30) but does not affect the photochemistry of the PHR domain (31).

There is compelling evidence that plant cryptochromes are phosphorylated upon photoexcitation, in particular in the CCE (32), and that this phosphorylation is required for the biological activity of these photoreceptors (32–35). \textit{Atcry1} (36, 37) and other cryptochromes (38) bind ATP, even though \textit{Atcry1} lacks a canonical nucleotide-binding site, as revealed by the co-crystal structure of the \textit{Atcry1} PHR domain (\textit{Atcry1PHR}) with the ATP analog AMP-PNP. In this structure, the adenine ring is sandwiched between Tyr-402 and Leu-296 within the active-site cleft, which is the DNA lesion-binding site in photolyases, the phosphates are exposed to the solvent, and hydrogen bonds are formed between $\beta$-phosphate and Arg-360 (37). \textit{Atcry1} and other cryptochromes have autophosphorylation activity \textit{in vitro} (34, 36, 38), but this activity does not explain the complete phosphorylation pattern of cry1 and cry2 (35).

ATP binding to \textit{Atcry1} also accelerates photoreduction of FAD$^{\alpha}$ to FADH$^+$. Transient ultraviolet absorption spectroscopy has revealed that the $pK_a$ of Asp-396, the proton donor for the anionic flavin radical, can be shifted to values of $>$9 by ATP binding, thus stabilizing the FAD$^+/\text{TrpH}^+$ radical pair close to the neutral Asp-396 long enough for further electron transfer and FAD protonation (31). A \textit{Chlamydomonas reinhardii} CPH1 cryptochrome mutant, in which the proton-donating Asp is replaced by Cys, forms only the anionic radical. ATP stabilizes FADH$^+$ in wild type and FAD$^{\alpha}$ in the mutant, which demonstrates that ATP prolongs the lifetime of the signaling state (20).

Another role of ATP was discovered in Trp-triad mutants of \textit{Atcry1} and \textit{Atcry2}, whose FAD$^{\alpha}$ is not photoreduced \textit{in vitro}. ATP binding partially restores photoreduction in these mutants, which can be explained by stabilization of the flavin semi-reduced state (39, 40) and an alternative electron transfer pathway (22).

We previously identified an \textit{Atcry1} mutant with a Leu $\rightarrow$ Phe exchange at position 407 (\textit{cry1L407F}) near the ATP-binding site. Plants that carry this dominant mutation flower early under non-inducing short-day conditions; they are hypersensitive to blue, red, and far-red light; and show a constitutive photomorphogenic (\textit{cot})-like phenotype. Results of molecular dynamics simulations suggest that \textit{cry1L407F} mimics an ATP-bound state of cry1 or promotes ATP binding (41) and thus could accumulate higher levels of FADH$^+$. Here, we analyzed whether ATP binding is affected in the \textit{cry1L407F} mutant. Moreover, we investigated whether ATP binding is a common feature of plant cryptochromes and how a recently discovered chemical inhibitor of \textit{Atcry1}, 3-bromo-7-nitroindazole (42), blocks \textit{Atcry1} function.

Results

\textbf{Adenosine phosphates enhance photoreduction of \textit{Arabidopsis} cry1 wild type and the \textit{cry1L407F} mutant proteins}

It has previously been shown that addition of ATP to crychromes \textit{in vitro}, including the shorter PHR domain (\textit{Atcry1PHR}), leads to both pronounced photoreduction of FAD$^{\alpha}$ (seen as a decrease in absorbance at 450 nm) and levels of this redox state lower than those of controls without added ATP (22, 31, 43). However, such a profound effect of ATP was not observed for full-length \textit{Atcry1} in the study of Gao \textit{et al.} (19). To determine whether the hyperactive \textit{cry1L407F} mutant indeed has a response to ATP different from the wild type, we measured its photoreduction in the absence and presence of ATP, ADP, and non-hydrolyzable AMP-PNP added in 100-fold molar excess over the protein. We used only the PHR domains (residues 1–509) of the \textit{cry1} PHR domains were expressed at much higher levels in \textit{Escherichia coli} and reached a higher purity compared with expression of full-length \textit{cry1} in insect cells (Fig. 1) (19). The \textit{cry1} PHR domain has essentially the same spectroscopic behavior as full-length \textit{cry1} carrying the flexible CCE (31). For simplicity, however, we will refer to these proteins as \textit{cry1WT} and \textit{cry1L407F}.

Consistent with previous reports, ATP caused a strong acceleration of photoreduction of \textit{cry1WT}, and the concentration of FAD$^{\alpha}$ remaining after prolonged exposure to blue light was significantly lower than that of the control without ATP (Fig. 2A). The curves of the decrease in absorbance at 450 nm over time (Fig. 2A) and the concomitant increase in absorbance at wavelengths above 500 nm (Fig. 3) indicated that, under steady-state conditions, FAD$^{\alpha}$ is transformed only to the FADH$^+$ species. Under blue light irradiation, the calculated half-life of FAD$^{\alpha}$ in the wild-type protein without added ATP was 31.4 $\pm$ 2.7 min. In presence of ATP, the curve for \textit{cry1WT} could be fitted only with two components, with $t_{1/2}$ = 0.9 $\pm$ 0.1 min and $t_{1/2}$ = 18.7 $\pm$ 2.2 min. All other photoreduction kinetics showed a monoeponential behavior. ADP had nearly the same effect as ATP, whereas AMP-PNP was slightly less effective (Fig. 2A). In
contrast to photoreduction of Atcry2 (22), Atcry1 photoreduction was not affected by the metabolites NADP and NADPH, which also contain the ADP moiety (Fig. 2B). As with the wild-type protein, FADox photoreduction of cry1L407F was accelerated in the presence of ATP, but the effect was less pronounced ($t_{1/2} = 99.1 \pm 15.3$ min without ATP and $t_{1/2} = 34.8 \pm 2.3$ min in the presence of ATP), and the level of FADox remaining after 180 min of photoreduction (Fig. 2C) was accordingly higher than in cry1WT. Surprisingly, the ATP analog AMP-PNP did not stimulate photoreduction of cry1L407F (Fig. 2C). This suggests that replacement of Leu by Phe at position 407 causes a structural change that affects the binding mode and/or affinity of ATP and AMP-PNP. Similar to the wild-type protein, photoreduction of cry1L407F was essentially unaffected by the addition of NADP or NADPH (Fig. 2D).

The steady-state level of FADH$^+$ in cry1 not only depends on the kinetics of light-driven FADox → FADH$^+$ conversion but also on FADH$^+$ → FADox dark reversion. We therefore tested the re-accumulation of FADox in cry1WT and the cry1L407F mutant. Samples were exposed to saturating blue light and then kept in the dark. The kinetics of FADH$^+$ → FADox dark reversion were monitored spectroscopically (Fig. 3) and were faster for the wild type in the absence of ATP (cry1WT $t_{1/2} = 98$ min; cry1L407F $t_{1/2} = 170$ min). When ATP was added, the dark recovery of both proteins was delayed, and this was more pronounced for cry1L407F (cry1WT $t_{1/2} = 104$ min; cry1L407F $t_{1/2} = 233$ min). These photoreduction experiments clearly showed a positive effect of ATP and ADP on the formation of FADH$^+$ in both cry1WT and cry1L407F. However, the hyperactivity of the cry1L407F mutant cannot be rationalized by a higher level of FADH$^+$ under steady-state blue light conditions.

Tyrosine at position 402 is required for ATP binding by cry1

The structure of the PHR domain of cry1 soaked with AMP-PNP (PDB entry 1U3D) showed that AMP-PNP binds in the FAD pocket, with the adenine ring sandwiched between Tyr-402 and Leu-296 (37). We reasoned that mutation of Tyr-402 to Ala should interfere with ATP binding. Accordingly, we constructed this mutant, expressed the protein in E. coli, and analyzed ATP binding of cry1WT and the cry1Y402A mutant by isothermal titration calorimetry (ITC). From the ITC data (Fig. 4), we calculated a dissociation constant ($K_D$) of 3.3 ± 1.6 μM for ATP binding to cry1WT, with a 1.1:1.0 stoichiometry of ATP to cry1, and a $K_D$ of 50.4 ± 28.0 μM for ATP binding to the cry1Y402A mutant, with a 0.85:1 stoichiometry of ATP to cry1Y402A. However, the $K_D$ value for cry1Y402A might be even larger because the mutant protein could not be saturated with ATP. The $K_D$ value obtained here for the cry1WT PHR domain is close to that obtained by ITC for full-length cry1 (38). We therefore conclude that the CCE, which is lacking in the cry1WT PHR domain, does not affect ATP binding and that Tyr-402 is important for ATP binding, as indicated previously in the cry1PHR/AMP-PNP co-crystal structure (37). We confirmed the low ATP-binding affinity of cry1Y402A in photoreduction experiments, in which the efficiency of photoreduction and levels of remaining FADox did not differ between samples with and without ATP (Fig. 5). ITC experiments done for the cry1L407F mutant confirmed ATP binding with a $K_D$ of 9.6 ±
1.7 μM and a 0.91:1 stoichiometry of ATP to cry1L407F (Fig. 4B). Thus, the affinity of cry1L407F to ATP is lower than that of wild type but sufficiently high under physiological conditions (see below).

**Adenosine phosphates cause conformational changes in cry1**

Binding of ATP to cryptochromes increases the light-induced yield of flavin radicals (20, 31), constrains dark recovery (21, 39, 40), and stabilizes the protein (39). We set out to determine whether metabolites affect structural changes of cry1L407F differently from that of cry1WT using partial trypsin proteolysis and thermo-FAD assays; in the latter, melting of the proteins was determined by following the release of FAD from dark-adapted and photoreduced (blue light-treated) proteins by monitoring the increase in FAD fluorescence.

In the proteolysis assays, both dark-adapted and blue light-treated wild type (Fig. 6A) and cry1L407F (Fig. 6B) proteins were partially protected against trypsic digestion by ATP, ADP, and AMP-PNP and, in case of wild type, slightly protected by NADP, NADPH, NAD, and NADH. To confirm that direct binding of ATP, ADP, or AMP-PNP caused this stabilization, we also analyzed the cry1Y402A mutant, which has a much lower affinity for ATP than the wild type. Indeed, this cry1 mutant was not protected against the protease by adenosine
phosphates or by any other metabolites tested (Fig. 6C). We analyzed the cryptochrome-specific effect of ATP on cry1 by analyzing DNA photolyase of *E. coli* (*Ec*PHR). *Ec*PHR was much more sensitive to trypsin than cry1, and ATP did not protect *Ec*PHR against the protease (Fig. 6D).

In addition to the partial proteolysis assays, we applied thermo-FAD assays. Wild-type cry1 had a melting temperature (*T*ₘ) of about 60 °C in the absence of ATP and a *T*ₘ of about 75 °C in the presence of ATP (Fig. 7A). Dark-adapted and photoreduced samples did not differ, except for a minor increase in melting temperature of photoreduced protein in the absence of metabolites. ADP and AMP-PNP had an effect on cry1WT similar to ATP, whereas nicotinamide adenine dinucleotides were less but still significantly effective (Fig. 7A). Compared with cry1WT, cry1L407F has slightly lower melting temperatures in the absence of metabolites, but it also responded to metabolites, in particular ATP, ADP, and AMP-PNP (Fig. 7B).

To confirm the reliability of the results of the thermo-FAD assays, we repeated the assays with EcPHR, which was not stabilized by ATP in the partial proteolysis assay. As expected, neither ATP nor any other tested metabolite showed binding to EcPHR (Fig. 8), which has a lower *T*ₘ (47 °C) than cry1WT (59 °C) and cry1L407F (57 °C).

**Nucleotide binding of cry1 is specific for adenosine phosphate**

The metabolites tested in the above-described photoreduction and stability assays, i.e., ATP, ADP, AMP-PNP, NAD(H), and NADP(H), all contain an adenine ring. We chose these compounds because the crystal structure of the PHR domain of cry1 with AMP-PNP revealed that the adenine moiety is perfectly sandwiched between Tyr-402 and Leu-296 and makes two hydrogen bonds with its β-phosphate to the surface-positioned Arg-360 (37). To determine whether the adenine ring or the β-phosphate contributes to the binding, we also tested the effect of CTP, GTP, and TTP on cry1WT and cry1L407F and cry1Y402A mutants in thermo-FAD assays (Fig. 9). cry1WT and cry1L407F were strongly stabilized by ATP (∆*T*ₘ ~18 °C); GTP (∆*T*ₘ ~7 °C) moderately stabilized and TTP (∆*T*ₘ 4 °C) and CTP (∆*T*ₘ 2 °C) only slightly stabilized these proteins. In general, purine nucleotides probably fit better in the flavin-binding pocket than pyrimidine nucleotides. The cry1Y402A mutant did not respond to ATP or any other nucleotide triphosphate, as expected from the ITC data.

**Chemical inhibitor of cry1 interferes with ATP binding**

Ong *et al.* (42) recently showed that 3-bromo-7-nitroindazole (3B7N) specifically inhibits cry1-controlled blue light responses, such as the inhibition of hypocotyl growth under blue light, and provided evidence that 3B7N binds to cry1-pro-
duced in an in vitro expression system. However, the binding mode of 3B7N to cry1 remained unsolved. We reasoned that 3B7N could interfere with the binding of ATP to cry1. We therefore tested whether 3B7N has a similar effect as ATP in thermo-FAD assays, i.e. whether it causes an increase in the melting temperature of cry1. When 3B7N was in 10-fold molar excess over the protein, the melting temperature of cry1WT increased by 8.7 °C, which is almost identical to the effect exerted by the same concentration of ATP (Fig. 10A). It is important to note that due to the low solubility of 3B7N in aqueous solution, this compound and ATP were provided at only a 10-fold molar excess above the protein. Thus, at least the ATP concentration was not saturated, and Fig. 10A cannot be directly compared with Figs. 7 and 9, where the molar excess of ATP was 100-fold. Interestingly, when ATP and 3B7N were added together, each at a 10-fold molar excess over the protein, the melting temperature increased by 8.2 °C, which is very close to the increase caused by each compound alone (Fig. 10A).

These data suggest that 3B7N competes with ATP for the same binding site of cry1. To test this more rigorously, we assayed cry1Y402A under identical conditions; this mutant protein binds ATP much more weakly than cry1WT. Indeed, neither ATP nor 3B7N affected the melting temperature of cry1Y402A (Fig. 10A). Competitive binding of 3B7N might also reduce the occupancy of cry1 with ATP in planta. To analyze whether 3B7N interferes with the photoreduction of cry1, we irradiated cry1WT and cry1Y402A with blue light in the absence and presence of 3B7N and monitored the formation of FADH° spectroscopically (Fig. 10, B and C). 3B7N clearly inhibited the accumulation of FADH°. Because this effect was observed only for cry1WT and not for the cry1Y402A mutant, this inhibitory effect of 3B7N is specific and requires direct binding of this compound to cry1. This conclusion is supported by a molecular docking model (Autodock Vina), in which 3B7N is accommodated in the cry1 nucleotide-binding site where the adenine ring of ATP would be positioned (Fig. 10D).

Figure 6. Partial tryptic digestion of cry1WT, cry1 mutants, and E. coli photolyase. Representative Coomassie-stained gels (from three replicates) show the results for cry1WT (A), cry1L407F (B), cry1Y402A (C), and E. coli photolyase (D). A and B, protein samples were dark-adapted or pre-illuminated with blue light until FAD° photoreduction was complete. C and D, protein samples were dark-adapted. Protein concentration, 10 μM; metabolite concentration, 1 mM; and trypsin concentration, 10 μM. Reactions were incubated at 30 °C for 30 min under a red safelight. M, protein markers; I, input; —, no metabolite. Protein bands were scanned, quantified by ImageJ, and normalized against the input (I) set as 100%.

Figure 7. Thermo-FAD assays showing binding of adenosine phosphates to cry1. A, cry1WT, and B, mutant cry1L407F. Proteins were either dark-adapted or pre-illuminated with blue light (photoreduced) until FAD° photoreduction was complete, as indicated. Protein concentration, 10 μM; metabolite concentration, 1 mM. Melting points were calculated from the peak fluorescence value of released FAD seen in melting curves as exemplified in Fig. 8. Given are means and standard deviations of three independent replicates.
We tested more specifically binding of 3B7N to cry1WT by ITC and calculated from these data a $K_D$ of $50 \pm 29 \mu M$ with a 0.82:1 stoichiometry of 3B7N to cry1WT (Fig. 11). Because a binding curve could be fitted from the injection heats, this binding is specific although lower than that of ATP.

**Discussion**

This study was undertaken to elucidate why the cry1L407F mutant has stronger biological activity than the wild-type protein cry1 and to investigate more generally the role of ATP binding to cry1. The cry1L407F mutant allele was previously identified in a suppressor screen of a late-flowering line (41). This mutant is hypersensitive toward blue, red, and far-red light (41). This phenotype is not caused by conditions, and this mutant is hypersensitive toward blue, red, and far-red light (41). This phenotype is not caused by the PHR domain of cry1 (31, 43), ATP, ADP, and AMP-PNP strongly enhanced FAD photoreduction of cry1PHR (Figs. 2, 3, and 5). The previously observed lack of effect of ATP on the photoreduction of full-length cry1 (19) could in principle be caused by the CCE hindering the accessibility of the ATP-binding site. However, this possibility was excluded when we determined a $K_D$ of 3.3 $\mu M$ for ATP binding for the cry1WT PHR domain, which is essentially the same as that determined for full-length cry1 ($K_D = 4.2 \mu M$) by ITC (38). Moreover, we checked binding of AMP-PNP to cry1WT PHR and full-length cry1 by thermo-FAD assays and found a similar behavior for the two proteins except that full-length cry1 had a higher melting temperature than the truncated cry1 in the absence of the ligand (Fig. 12). Chromatographic methods yielded a higher $K_D$ of 19.8 $\mu M$ for full-length cry1 and a stoichiometry of 0.4 ATP to 1.0 cry1 (36), and a $K_D$ of 75 $\pm 22 \mu M$ with a stoichiometry of 1 for AMP-PNP binding to cry1PHR (37). The lower affinity of cry1 to AMP-PNP than to ATP could be the reason why AMP-PNP was less effective than ATP in accelerating cry1 photoreduction (Fig. 2) and why the increase in the melting temperature in the thermo-FAD assay was less pronounced (Fig. 7).

We chose to study the photosensory photolyase-related (PHR) domain of cry1 instead of the full-length cry1 proteins as PHR alone has biological activity (44) and a similar photochemistry as full-length Atcry1 (31). Thus, we are convinced that our conclusions based on PHR are also valid for full-length cry1, which carries the flexible CCE (45, 46).

Consistent with earlier studies on Atcry2 (22) and other cryphotochromes, including the PHR domain of cry1 (31, 43), ATP, ADP, and AMP-PNP strongly enhanced FAD photoreduction of cry1PHR (Figs. 2, 3, and 5). The previously observed lack of effect of ATP on the photoreduction of full-length cry1 (19) could in principle be caused by the CCE hindering the accessibility of the ATP-binding site. However, this possibility was excluded when we determined a $K_D$ of 3.3 $\mu M$ for ATP binding for the cry1WT PHR domain, which is essentially the same as that determined for full-length cry1 ($K_D = 4.2 \mu M$) by ITC (38). Moreover, we checked binding of AMP-PNP to cry1WT PHR and full-length cry1 by thermo-FAD assays and found a similar behavior for the two proteins except that full-length cry1 had a higher melting temperature than the truncated cry1 in the absence of the ligand (Fig. 12). Chromatographic methods yielded a higher $K_D$ of 19.8 $\mu M$ for full-length cry1 and a stoichiometry of 0.4 ATP to 1.0 cry1 (36), and a $K_D$ of 75 $\pm 22 \mu M$ with a stoichiometry of 1 for AMP-PNP binding to cry1PHR (37). The lower affinity of cry1 to AMP-PNP than to ATP could be the reason why AMP-PNP was less effective than ATP in accelerating cry1 photoreduction (Fig. 2) and why the increase in the melting temperature in the thermo-FAD assay was less pronounced (Fig. 7).

It was proposed earlier that ATP binding has a stabilizing effect on cry1 based on molecular dynamics simulations of the cry1WT PHR domain and the cry1L407F mutant (41) and on partial proteolysis assays (39). We reasoned that the photochemistry of the cry1L407F mutant could be affected, and we hypothesized that this change in the protein could promote the formation of the signaling state (most likely via accumulation of FADH+) or cause a slower dark reversion than cry1WT. In our assay of the kinetics of dark reversion, the FADH+ $\rightarrow$ FADox conversion of cry1WT had a half-life of $t_{1/2} = 98$ min that was essentially not affected by ATP. In contrast, dark reversion of cry1L407F was slower in the absence of ATP ($t_{1/2} = 170$ min) and further retarded by ATP ($t_{1/2} = 233$ min). Under physiological conditions where ATP is present, cry1L407F should therefore
stay active longer than wild type when light is switched off. However, under steady-state blue light conditions $\text{cry1}_{L407F}$ accumulated less FADH° in the absence and presence of ATP. Thus, the hyperactivity of $\text{cry1}_{L407F}$ cannot be linked to the amount of flavin's lit state. The dark reversion of $\text{cry1}_{\text{WT PHR}}$ measured here is much slower than the few minutes reported for the full-length At$\text{cry1}$ in air-saturated solution (47). This difference could be caused by lower oxygen tension in our assays, temperature, buffer effects, or the absence of the CCE. Because we compared wild-type and mutant PHR domains under identical conditions, we consider that this difference is not relevant for the interpretation of our data.

It has been suggested that the $\text{cry1}_{L407F}$ mutation might promote ATP binding (41), but ITC measurements performed here demonstrate that the $K_D$ value is even higher than that of wild type (Fig. 4). This result is consistent with the fact that photoreduction of $\text{cry1}_{L407F}$ is less enhanced by adenosine phosphates compared with cry1WT (Fig. 2), and its melting temperature in the absence and presence of ATP was lower than that of cry1WT (Fig. 7). The latter result was even unexpected as the molecular dynamics simulation predicted that the structural flexibility $\text{cry1}_{L407F}$ is reduced. Taken together, our data showed that the hypersensitivity of $\text{cry1}_{L407F}$ is not caused by elevated FADH° accumulation or by promotion of ATP binding. At first glance, these data suggest that the activity of $\text{cry1}$ is not correlated with the level of formed FADH°. However, the $\text{cry1}_{L407F}$ mutant not only is hypersensitive to light but also has a constitutive photomorphogenic (cop) phenotype, with hypocotyl growth inhibition in the dark (41). Thus, the $L407F$ mutation might cause a structural change that mimics the active signaling state of $\text{cry1}$. In cry1WT, this signaling state can be achieved only by FADH° formation and, possibly, ATP binding. A behavior similar to that of $\text{cry1}_{L407F}$ has observed in a Trp-triad (W400A) $\text{cry1}$ mutant, which produces high levels of anthocyanin in the dark and constitutively interacts with constitutive photomorphogenic 1 (19); such responses in the wild type are light-induced. $\text{cry1}_{L407F}$ and $\text{cry1}_{W400A}$ mutants thus cannot provide insight into the light-dependent...
ATP binding of plant cryptochrome

Figure 11. Binding of 3B7N to wild-type cry1 determined by isothermal titration calorimetry. The top panels show the injection heats; the bottom panels show the calculated binding curve fitted to the measured integrated areas of the injection heats and the molar ratio. The fits were calculated using the program Origin.

Figure 12. Thermo-FAD assays showing the response of the cry1WT PHR domain and cry1WT full-length protein to AMP-PNP. Dark-adapted proteins were used at a final concentration of 10 μM; AMP-PNP concentration, 1 mM. Melting points were calculated from the peak fluorescence value of released FAD seen in melting curves as exemplified in Fig. 8. Given are means and standard deviations of three independent replicates.

activation mechanism of cry1, but they might resemble a structure similar to the signaling state. Unfortunately, our attempts to crystallize cry1407F for X-ray analysis have not yet been successful as a structure of full-length cry1 is also missing. Thus, a structure of the signaling state of cry1 or of a mutant protein that mimics this state remains to be determined in the future.

Blue light-induced phosphorylation of cry1 in planta has been reported (34); part of this might be due to autophosphorylation, as observed for recombinant proteins in vitro (34, 36, 38). The phosphorylated form of cry1 resembles the active state of this photoreceptor (34). The role of ATP on cry1 photoreduction and stabilization reported in our study is, however, not related to the autokinase activity of cry1 because non-hydrolyzable ADP and AMP-PNP had effects similar to those of ATP (Figs. 2 and 6–8). The adenine ring of ATP was required to obtain the strongest responses; GTP had only a moderate effect, and the pyrimidines CTP and TTP had essentially no effect (Fig. 9). These results are consistent with the AMP-PNP/cry1 co-crystal structure, which reveals the perfect sandwiched position of the adenine ring of AMP-PNP between Tyr-402 and Leu-296 and hydrogen bonding between the N6 amino group and Asp-409 (37). GTP would not be able to interact similarly and would not fit properly in this binding site because of a steric clash between Leu-398 and its N2 amino group. Binding of adenine by cry1 differs strikingly from the nucleotide-binding mode of DNA photolyases, which accommodate either pyrimidine dimers or even a single pyrimidine (48) in an edge-on fashion in the catalytic pocket. The lack of ATP binding by photolyase was confirmed by the results of our thermo-FAD assays, which showed that ATP had no effect on EcPHR (Fig. 8).

We also investigated the effect of nicotinamide adenine dinucleotides on photoreduction of the FADox cofactor and the stability of cry1. These compounds all contain the ADP structure and were previously shown to promote photoreduction of full-length Atcry2 (22). In our study, these compounds did not have any effect on cry1 photoreduction and had only minor effects in proteolysis and melting temperature assays when added at the same concentration as ATP (Figs. 2, 6, and 7). The reason for this difference between cry1 and cry2 is not yet clear. In ITC assays, ATP binding to full-length Atcry2 has a K_D of 0.9 μM (38), but with a chromatographic method, a significantly higher K_D of 49 ± 12 μM has been reported (22). It is therefore difficult to even speculate whether cry2 has a more flexible or wider binding pocket than cry1 that allows the binding of bulkier ADP derivatives.

As the chemical compound 3B7N is a specific inhibitor of cryptochrome signaling in Arabidopsis and binds to cry1 produced in a wheat germ system (42), we tested whether 3B7N binds to recombinant cry1WT and interferes with the binding of ATP to cry1. ITC assays confirmed binding of 3B7N to cry1 (Fig. 11), although the affinity is significantly lower than that of ATP. Likewise binding of 3B7N to cry1 was seen in thermo-FAD assays, and the results were essentially the same as with ATP alone (Fig. 10A). Thermo-FAD assays also revealed that 3B7N has no effect on cry1Y402A, which also does not respond to ATP (Fig. 10A). Thus, 3B7N and ATP bind at the same site of cry1 and compete for binding, as also indicated by molecular docking (Fig. 10D). Because 3B7N hinders the formation of FADH’ in cry1WT (Fig. 10B) but not in cry1Y402A (Fig. 10C), and inhibits cry1 function in planta (42), we conclude that cry1 requires neutral semi-reduced FAD for biological activity and that ATP promotes this process.

If one considers the millimolar concentration of ATP within a cell (49), it is at first glance surprising that 3B7N at 2.5 μM in the medium (42) competes with ATP at the binding site of cry1. We assume that 3B7N is taken up actively by the plant cell and reaches concentrations within the cell higher than in the medium.

3B7N is a potent inhibitor of neuronal nitric-oxide (NO) synthase (50). Treatment of plants with NO donors mimics light responses, which suggests that NO might act as a positive reg-
ulator in light-signaling cascades (51). However, the originally postulated NO synthase NOS1 in Arabidopsis was later shown not to have NOS activity but instead functions as a GTPase (52, 53). Thus, there is no clear evidence for enzymatic production of NO in plants. Moreover, an indirect role of 3B7N on cry-mediated responses via NO regulation has been excluded (42). We therefore believe that the negative effects of 3B7N on blue light responses in Arabidopsis can be explained by its inhibitory role in ATP binding to cry1 and formation of FADH•

In view of the micromolar dissociation constant for ATP binding by cry1 and a cytosolic ATP concentration in the millimolar range (49), it is very likely that most of the cryptochrome molecules are occupied with ATP. Thus, it is not evident how ATP itself could have a regulatory role for cry1 activity, even though ATP stabilizes the FAD radical state (20, 31, 43) and is required for autokinase activity (34, 36, 38). Along these lines, we also do not think that cry1 senses the energy status of the cell by measuring the ATP/ADP ratio because both ATP and ADP bind to cry1.

The ATP and 3B7N binding characteristics of cry1 observed in our study are most likely not specific features of this ortholog of the cryptochrome/photolyase family. Clustering of sequence similarity networks of the entire family (February, 2017) revealed that 417 orthologs co-cluster as plant-type cryptochromes (pCry) with cry1 and cry2 from A. thaliana (Fig. 13A). Interestingly, all these cryptochromes belong to the green lineage, including orthologs from other streptophytes and also from green algae. For example, like cry1, CPH1 from C. reinhardtii binds ATP and self-phosphorylates in vitro (21). Other chlorophytes with this type of ATP binding and plant-like cryptochrome include Coccomyxa subellipsoidea (Uniprot entry I0YVL3), Volvox carteri (D8UF46), Gonium pectoral (A0A150G7B8), Auxenochlorella protothecoides (A0A0875LJ0), and Monoraphidium neglectum (A0A0D2L2I6). In the pCry subfamily, the residues that constitute the ATP-binding site itself are almost strictly conserved (Fig. 13B). The residue that packs to the adenine moiety of ATP, Tyr-402, is replaced by phenylalanine only in cry1b orthologs from Solanum tuberosum and Solanum lycopersicum (Uniprot: Q93VS0, M1AG20) (54). Likewise, Asp-409, which lies to next the N6 amino group, is highly conserved, as is the cluster of basic residues surrounding the β- and γ-phosphate of ATP (Arg-239, Arg-240, Lys-292, Arg-360, and Lys-425). β- and γ-phosphates support the binding, as seen from the lower effect of AMP in the thermo-FAD assay (Fig. 14). Interestingly, the subfamily of pCry-related orthologs, of which CryP from the diatom Phaeodactylum tricornutum has been characterized, lacks the conserved set of residues in the ATP-binding site. This restricted distribution of pCry orthologs in the green lineage hence indicates that ATP binding as a “booster” for light sensitivity arose only in this branch of photosynthetic organisms. Given that pCry orthologs apparently exist in vivo predominantly as ATP complexes, it cannot be excluded that the bound ATP molecule is also involved in the interaction with other downstream effector molecules.

### Experimental procedures

#### Cloning of expression constructs

For the cloning of the PHR domain (residues 1–509) of CRY1WT and cry1L407F, the following primers were used: 5′-GAA TTC GTC TGG TTC TGT ATC TGG TTG TG-3′ and 5′-GCC GCC GCT TAT CCE TCT TCG GAT-3′. The full-length coding sequence of CRY1WT in a pBluescript SK plasmid was used as a template for PCR. Plasmid pENTR/D carrying the full-length coding sequence of cry1L407F served as template for the expression construct of cry1L407F. PCR products were identified and purified via agarose gel electrophoresis, digested with NolI and EcoRI, and inserted into the NolI and EcoRI restriction sites of the expression vector pACYCDuet-1 (Novagen). The correctness of the sequences of the final expression constructs was verified by sequencing.

#### Site-directed mutagenesis

To introduce the Y402A substitution into the CRY1 gene, we used the QuickChange® II site-directed mutagenesis kit (Agilent Technologies) with the following primers (mutated codons are in lowercase letters): 5′-GAT GCT CTT GTG TGG CAA gcg ATT ACC GGT ACT CTC CCG G-3′ and 5′-CGG GAG AGT ACC GTG AAT cgc TTG CCA ACC AAG AGC ATC G-3′. The expression construct pACYCDuet CRY1WT was used as a template. The final expression construct was verified by sequencing.

#### Expression and purification of recombinant proteins

For the overproduction of recombinant proteins, competent BL21(DE3) (New England Biolabs) cells were freshly transformed and plated on LB-agar plates containing chloramphenicol (34 µg ml⁻¹). Plates were incubated at 37 °C overnight. A single colony was used to inoculate 50 ml of LB medium containing chloramphenicol in a 100-ml flask, and this pre-culture was incubated at 37 °C with shaking (200 rpm) overnight. Six flasks with 50 ml of LB medium containing chloramphenicol were inoculated with 2 ml of the pre-culture and were incubated for 6 h at 37 °C with shaking at 200 rpm; these cultures were used to inoculate six 3-liter baffled flasks, each containing 1.5 liters of LB medium and chloramphenicol. Cultures were incubated at 37 °C with shaking at 140 rpm to an A600 of ~0.4. The temperature was then lowered to 25 °C, and the A600 of the cultures was monitored until they reached 0.6–0.8. At this point, formation of the recombinant cryptochromes was induced by adding IPTG to a final concentration of 1 mM. After 14 h of incubation, growth was stopped by placing the bacterial cultures on ice. The cells were then collected by centrifugation (5,000 g, 4 °C, 20 min). The pellets were resuspended in pre-cooled phosphate-buffered saline, pH 7.4, and the suspension was transferred to Falcon tubes and centrifuged (4,000 × g, 4 °C, 45 min), and pellets were stored at ~80 °C.

For the purification of the recombinant proteins, ~30 g of bacterial pellets harvested from 18 liters of culture was resuspended in 100–120 ml of pre-cooled lysis buffer (100 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole, and 10 mM β-mercaptoethanol). All subsequent steps were carried out under red light. The cells were disrupted...
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A

B
by a French press, and the bacterial lysate was pelleted by cen-
trifugation (40,000 × g, 4 °C, 60 min). The resulting superna-
tants were passed through 0.45-μm filters (Millipore) and
subsequently loaded onto a 1-ml nickel-nitrioltriacetic acid
column using the ÄKTApurifier 100 plus system (GE Healthcare).
Bound proteins were eluted with a linear imidazole gradient
(10–500 mM, 30 column volumes), and 1-ml fractions were
collected by a Frac-950 fraction collector (GE Healthcare). The
purities of the proteins of interest present in the collected frac-
tions were checked by SDS-PAGE and Western blot analysis.
Fractions containing the recombinant protein were pooled,
diluted 1:10 with protein storage buffer (10 mM Tris-HCl, pH
8.0, 200 mM NaCl, 10% glycerol (v/v), and 10 mM β-mercapto-
ethanol), and concentrated to about 1 ml by ultrafiltration
(Amicron 30-kDa MWCO; Millipore). This solution was
directly loaded onto a size-exclusion column, and proteins were
isocratically eluted using protein storage buffer and the ÄKTA-
purifier system also removing any remaining imidazole. The
eluates were collected in 1-ml fractions, and protein-containing
fractions were pooled and then concentrated by ultrafiltration.
Protein solutions were stored at −80 °C.

**Construction of *E. coli* photolyase (EcPHR) expression plasmid, protein expression, and purification**

The complete coding sequence of EcPHR present in a
pQE-60 vector (Qiagen) with the C-terminal His6 tag was cut
out of the vector using Ncol and HindIII and inserted into the
Ncol and HindIII sites of expression vector pET-28a (Nova-
gen). The correctness of the sequence was verified by sequenc-
ing. EcPHR expression in *E. coli* BL21(D3) in LB medium at an
A600 of ~0.6 was induced with 1 mM IPTG, and the culture was
further incubated at 30 °C for 10–12 h. EcPHR was purified as
described for *A. thaliana* cry3, including a heparin column step
that also removes the remaining imidazole (55) but omits the
size-exclusion chromatography step.

**UV-visible spectroscopy and photoreduction**

UV-visible spectra of the proteins in an estimated concentra-
tion of 10 μM were measured in a double-beam spectrophotom-
eter (UV-2401 PC, Shimadzu) at a range of 220–700 nm at
10 °C. For photoreduction experiments, a baseline with protein
storage buffer was measured. All samples were prepared and
transferred to cuvettes under red light, and the spectra were
recorded against the reference buffer. As reductant, 1,4-dithio-
arethitol (DTT) was added to a final concentration of 20 mM. The
samples were exposed to blue light (450-nm interference filter
from Schott, 10-nm bandwidth, 50 μmol m−2 s−1), and spectra were
recorded at different time points. Metabolites (purchased from
Sigma, purities ≥98%) were used at a final concentration of 1 mM
in both the sample and the reference cuvette.

In photoreduction experiments with 3B7N (purchased from
Toronto Research Chemicals; dissolved in DMSO), 3B7N and
ATP were at a final concentration of 50 μM. Controls contained
the same concentration of DMSO (175 mM). The following buffer
was used in these experiments: 100 mM MOPS, pH 7.8, 400 mM
NaCl, 10% glycerol (v/v), and 20 mM β-mercaptoethanol.

**Pre-illumination of samples with blue light**

To compare samples pre-illuminated with blue light to dark
samples, a 300-μl solution of protein at 12.5 μM with 25 mM
DTT was prepared in protein storage buffer; the solution was
divided between two cuvettes (150 μl each). A baseline from
220 to 700 nm was recorded for a difference spectrum. The
illuminated sample was exposed for 3 h at 10 °C to blue light
(450-nm interference filter, 10-nm bandwidth; 50 μmol m−2
s−1, 10 °C); the dark sample was kept under the same conditions
in the reference cuvette holder. When no further reduction of
the FAD cofactor was observed, the illumination was stopped,
and the samples were promptly used for melting point analyses
and partial tryptic digestions.

**Melting point analysis**

The melting point of proteins was determined in thermo-
FAD assays using a Qiagen Rotor-Gene Q (56). 16-μl of pre-
illuminated and dark protein samples described above were
then pipetted into reaction tubes, and 4 μl of a metabolite
(stock solutions: 5 mM in protein storage buffer) was added, and
the solution was mixed by pipetting. In each 20-μl reaction,
the final concentration of protein was 10 μM. All metabolites were
used at a final concentration of 1 mM; 3B7N was used at a final
concentration of 100 μM (dissolved in DMSO, final DMSO con-
centration of 350 mM in assays). Mock controls contained
the same final concentration of DMSO. The melting points were
analyzed using a linear temperature increment ranging from 25

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**Figure 14.** AMP binds less efficiently to cry1 than ADP and ATP. cry1WT, 10 μM; adenosine phosphates, 1 mM. Mock control (−) contained the same final concentration of DMSO (350 mM) as the other reactions. Assays were performed in 100 mM MOPS, pH 7.8, 400 mM NaCl, 10% glycerol (v/v), and 20 mM β-mercaptoethanol. Given are means and standard deviations of three independent replicates.

**Figure 15.** ATP binding of plant cryptochrome

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**Figure 13.** ATP-binding site as a hallmark of cryptochromes of green-lineage organisms. **A**, sequence-similarity network of the cryptochrome/photolyase family (left) and the subfamily of plant-like cryptochromes (right). Only the well-characterized class I and II CPD photolyases, 6-4 photolyases/animal-like cryptochromes, DASH cryptochromes, and bacterial cryptochromes of the CryPro/Fe-S-BCP subfamilies are labeled. The plant-like cryptochromes are split into the pCry subfamily, which correspond to cry1 and cry2 (light green), and the pCry-related orthologs (light green), which are found in several algae, animals, and bacteria but lack the conserved residues of the ATP-binding site. **B**, structure of the ATP-binding site of cry1 (PDB entry 1U3D). Residues in contact with ATP are depicted as stick models. Position-specific sequence variation shown for these residues is based on 339 non-redundant orthologs of plant-like cryptochromes.
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to 99 °C. For each data point, the temperature was raised by 0.5 °C. The fluorescence (excitation wavelength 470 ± 10 nm; emission wavelength 510 ± 5 nm) was measured at each time point, and the denaturation of the protein was represented as the increase in fluorescence caused by the release and oxidation of the FAD cofactor. The fluorescence increment was then inversely plotted as a melting curve. The peak value of the melting curve was regarded as the melting point.

Partial tryptic digests and protein separation

The pre-illuminated and dark-adapted samples were each pipetted into reaction tubes (15 μl each). Then, 4 μl of a metabolite (stock solutions: 5 mM in protein storage buffer) was added. For samples without added metabolite, the same amount of protein storage buffer was used. All samples were incubated for 10 min on ice to allow binding of metabolites. Then 1 μl of trypsin (200 μM, from bovine pancreas, as L-1-tosylamido-2-phenyl ethyl chloromethyl ketone-treated trypsin, ≥10,000 benzyol-l-arginine ethyl ester units per mg of protein, Sigma) in 1 mM HCl and 20 mM CaCl₂ were added to each sample (final trypsin concentration 10 μM) and mixed by pipetting. Reference samples that were not treated with trypsin received 1 μl of 1 mM HCl containing 20 mM CaCl₂. The samples were incubated for 30 min at 30 °C under red light. Reactions were stopped by adding 5 μl of stop solution (5 mM EDTA and 5 mM PMSF in 5X SDS/sample buffer: 225 mM Tris-HCl, pH 6.8, 250 mM DTT, 5% SDS (w/v), 50% glycerol (v/v), and 0.05% bromphenol blue (w/v)), and samples were subsequently kept at 99 °C for 10 min. After centrifugation (20,000 g, 25 °C, 2 min), samples were loaded on pre-cast SDS-polyacrylamide gels with an acrylamide gradient of 4–15% (Mini-Protean system, 25 °C, 25 min). As receptor for docking, the cry1 AMP-PNP complex structure (PDB code 1U3D) was used after removal of endogenous water, ions, and AMP-PNP ligand. Partial charges were assigned to the protein according to the ff14sb force field and were set up for FAD in its oxidized state and for 3B7N by antechamber using the AM1-BCC model. The receptor search volume of 40 × 40 × 18 Å was centered around the ATP-binding site; docking of ligands 3B7N and, as reference, ATP, was analyzed using Autodock Vina (60) with default parameters (number of binding modes, 10; exhaustiveness of search, 8; maximum energy difference, 3 kcal/mol). The obtained docking poses were visualized using PyMOL, version 1.8.3.2 (61).

Author contributions—C. O. performed protein expression and purification, UV-visible spectroscopy, and limited proteolysis and thermo-FAD assays. N. N. expressed and purified proteins and carried out thermo-FAD and ITC experiments. C. O. and N. N. prepared the figures, wrote the figure legends and “Experimental procedures,” and analyzed the data. A. B. designed the experiments, analyzed the data, and wrote the manuscript. L.-O. E. analyzed the sequences and molecular docking, prepared the figures, and wrote part of the text. L. H. contributed to the overall concept and manuscript preparation.

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Molecular docking of 3B7N to the cry1 ATP-binding site

As receptor for docking, the cry1 AMP-PNP complex structure (PDB code 1U3D) was used after removal of endogenous water, ions, and AMP-PNP ligand. Partial charges were assigned to the protein according to the ff14sb force field and were set up for FAD in its oxidized state and for 3B7N by antechamber using the AM1-BCC model. The receptor search volume of 40 × 40 × 18 Å was centered around the ATP-binding site; docking of ligands 3B7N and, as reference, ATP, was analyzed using Autodock Vina (60) with default parameters (number of binding modes, 10; exhaustiveness of search, 8; maximum energy difference, 3 kcal/mol). The obtained docking poses were visualized using PyMOL, version 1.8.3.2 (61).

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References


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