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Searching for the Mechanism of Signalling by Plant Photoreceptor Cryptochrome

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ABSTRACT

Even though the plant photoreceptors cryptochromes were discovered more than 20 years ago, the mechanism through which they transduce light signals to their partner molecules such as COP1 or SPA1 still remains to be established. We propose that a negative charge induced by light in the vicinity of the flavin chromophore initiates cryptochrome 1 signalling. This negative charge might expel the protein-bound ATP from the binding pocket, thereby pushing off the C-terminus that covers the ATP pocket in the dark state of the protein. This conformational change should allow for phosphorylation of previously inaccessible amino acids. A partially phosphorylated 'ESSSSGRR-VPE' fragment of the C-terminus could mimic the sequence of the transcription factor HY5 that is essential for binding to the negative regulator of photomorphogenesis COP1. HY5 release through competition for the COP1 binding site could represent the long-sought connection between light activation of cryptochrome and modulation of photomorphogenesis.

Keywords: plant cryptochrome, photoreceptor, signalling, ATP, HY5, COP1

Introduction

Cryptochromes (CRYs) are >50 kDa large flavoproteins structurally derived from DNA repair enzymes photolyases. Plant and some animal CRYs act as photoreceptors, other CRYs were shown to act as light-independent transcription repressors [1, 2]. Upon irradiation by blue or near-UV light, the flavin adenine dinucleotide (FAD) cofactor in plant cryptochromes is reduced from the putative resting state, the fully-oxidized FAD (FAD_{ox}), to the putative signalling state, the semireduced neutral FADH[•] radical. This light-induced change in the redox state of FAD is suspected [3] to trigger a partial detachment of the flexible C-terminal domain (referred to as CCT: Cryptochrome Carboxyl Terminus) from the rigid N-terminal domain (referred to as PHR: the Photolyase Homology Region), which is harbouring the FAD cofactor. Loosening of the CCT is believed to be the initial signalling step, enabling plant CRY to transduce the light signal into a conformational signal that can be sensed by its partner molecules [3]. Nevertheless, it is unclear (i) how could a photoconversion of a neutral FAD_{ox} into an equally neutral FADH[•] trigger a substantial conformational change and (ii) what is the molecular mechanism by which the CCT interacts with its signalling partners.

Plant and some animal (e.g., human) CRYs were shown to bind ATP [4-7] and plant CRYs to undergo unspecified phosphorylation upon blue-light irradiation [5-7]. ATP is bound at the site equivalent to that of DNA binding to photolyases but there are no phosphorylatable amino acids in the vicinity of the ATP phosphates in the structure of plant CRY PHR [8]. Autophosphorylation is plausible at the CCT, provided that the CCT is in contact with the ATP-binding site at the PHR [9]. Crystal structure of a full-length plant CRY (including the CCT) is not available but the sequence analysis of *Arabidopsis thaliana* CRY1 (*AtCRY1*) reveals that its CCT contains 25 serine, 10 threonine and 4 tyrosine residues. Many of these residues are parts of patterns recognizable by common kinases and phosphatases and some of them are located next to positively charged arginines, which could be anchored to the negative phosphates of the bound ATP molecule prior to the light-induced repulsion of the CCT.

So far, the only attempt to propose a plausible mechanism of CCT detachment was made by the group of C. Lin [2,9] who suggested (contrary to the prevailing opinion [10-12]) that rather than FAD_{ox}, FAD^{•-} could be the resting state in photosensory CRYs. In analogy to photolyases, where FADH⁻ transfers an electron to the damaged DNA, the photoexcited [FAD^{•-}]* in CRY could transfer an electron to the ATP adenine and this would result in a cleavage of the gamma phosphate and drive phosphorylation of the CCT. The electron would then be transferred back to FAD (recovering the anionic FAD^{•-}) and the negative phosphates of the remaining ADP would electrostatically repel the phosphorylated CCT.

The electrostatic repulsion of the C-terminus upon phosphorylation seems plausible, nevertheless, the groups of D. Zhong and A. Sancar [13] have recently provided a strong argument against this mechanism by showing that the excited FAD^{•-} isoalloxazine is immediately quenched by intramolecular electron transfer (ET) to the FAD adenine in these proteins (within 12 ps, followed by back ET to the isoalloxazine in 2 ps). For comparison, the intermolecular ET from FADH⁻ in a photolyase to DNA takes about 250 – 300 ps [13,14]. In the light of these results, it is highly unlikely that the ET from [FAD^{•-}]* to ATP could compete with the ultrafast self-quenching process and a new reaction model needs to be suggested.

New model: repulsion of the C-terminus is triggered by a long-lived photogenerated charge in the FAD-binding pocket

Our latest experimental findings [15] indicate that as long as ATP is bound to plant CRY (and this is the likely situation *in vivo*: $K_{d,AtCRY1:ATP} \sim 1.4 - 20 \mu\text{M}$ [4,15,16], $c_{ATP} \sim 1.3 \text{ mM}$ in plant cells, [17]), FAD_{ox} photoreduction results in a formation of long-lived negative charge in the FAD-binding pocket [15,18]). Our data analysis implies that a dynamic protonation equilibrium $\text{D396}^- + \text{FADH}^{\bullet} \rightleftharpoons \text{D396H} + \text{FAD}^{\bullet-}$ is established, with the negative charge situated mostly on the aspartic acid D396 (~80%).

A recent study of another protein from the CRY/photolyase family, *Methanosarcina mazei* CPD II photolyase, in which the N5 atom of the FAD isoalloxazine is facing an asparagine instead of an aspartic acid, has shown that FAD^{•-} can get protonated by alternative pathways (presumably by solvent) to form FADH[•]; see Fig. 6 in [19]. This proton transfer occurs in 0.3 – 0.4 s as documented by Fig. 44B of [20].

Regardless of whether Asp^- or FAD^- is formed by photoreduction of FAD_{ox} , the resulting anion will live for a few hundreds of milliseconds prior to protonation [21], which is a long enough time for this photoinduced negative charge to cause conformational changes in the vicinity of the FAD cofactor [22] and to force the ATP molecule out of the binding pocket [23], which would rebound the whole CCT covering the binding pocket prior to the photoreaction (Fig. 1).

So far the only time-resolved study of light-induced CCT dissociation was published by Kondoh *et al.* [24], who compared the transient grating signal of full-length *AtCRY1* with that of its PHR. While the full-length protein showed a large diffusion signal indicative of conformational change in ~ 0.4 s (consistent with the estimated lifetime of the photogenerated negative charge [15,20]), this component was missing in the signal from the PHR sample. Based on our detailed knowledge of the protein purification procedure applied in this study and taking account of the high affinity of *AtCRY1* to ATP [15], we estimate that, at the very least, 30% of the full-length protein molecules still contained ATP. The observed transient grating signal indicating a conformational change of the CCT could hence well have been due to the protein fraction binding ATP.

In any case, plant CRYs should bind ATP *in vivo* [15]. It also seems established that their C-termini undergo a light-induced conformational change [24,25] and this change results in a multiple phosphorylation of the CCT in both CRY1 and CRY2 proteins [5-7,26]. A straightforward explanation of this phenomenon is that some of the previously inaccessible phosphorylation sites become available to protein kinases after the rebound of the CCT. We propose a good candidate for such sterically protected phosphorylation site is the 'STAESSSS' motif conserved in C-termini of both plant CRY1 and CRY2. In both cases, this motif is neighbored by positive amino acids (R629, R630 and R632 in *AtCRY1*; K554, K555, R557 and R562 in *AtCRY2*), which might be anchored to the phosphates of the PHR-bound ATP in the inactive state of the protein.

Mechanism of activation of the HY5 transcription factor: competitive binding of the plant CRY1 C-terminus to the regulatory protein COP1?

Interestingly, Partch *et al.* suggest that it is precisely the region around the 'ESSSS' motif, which undergoes structural rearrangement in response to light in *AtCRY1* CCT [25]. A closer look at the sequence reveals that this motif is neighbored by a 'VPE' fragment in *AtCRY1*. The 'VPE/D' fragment, preceded by four negatively charged amino acids, was identified as essential for binding of the HY5 transcription factor to its repressor COP1 ('Constitutive Photomorphogenic 1') [27]. We hence suggest that if two or three of the four neighbouring serine residues are phosphorylated, the 'ESSSSGRR---VPE' motif could well mimic the 'ESDEEIRRVPPE' sequence of HY5 [28], as illustrated by Fig. 2, and enable the substitution of HY5 at its COP1 binding site by the activated CCT and, consequently, also the binding of SPA1 (Suppressor of Phytochrome A) by CCT as proposed by Liu *et al.* [29]. The dissociation of the dark complex COP1-SPA1-HY5 induced by light-activated CCT would uncage HY5, which would then be free to promote the expression of genes known to be implied in the photomorphogenic development (Fig. 1).

Outlook

We have presented a new reaction model of how a light signal received by plant cryptochrome could be converted into a conformational signal that could be sensed by partner molecules downstream in the light-signalling pathway. The proposed repulsion mechanism could be generalized to all photosensory cryptochromes: to insect CRYs, where the CCT directly enters the pocket close to FAD [30-32] and where FAD_{ox} photoreduction leads to a long-lived FAD^- [12], as well as to red-light-absorbing aCRYs (animal-like CRYs [33]), where the photoconversion of FADH^+ to FADH^- might be the initial signalling step.

Ultimately, we would like to propose experiments that could corroborate or disprove our model:

- (i) It would be desirable to resolve the crystal structure of a full-length plant CRY (including CCT), both in the absence and in the presence of ATP. The crystal structures should reveal whether or not the 'STAESSSS' residues are accessible to kinases in the dark state of the protein.

HYPOTHESIS

(ii) The hypothesis that ATP is necessary to trigger the dissociation of CCT could be tested, *e.g.*, by comparing transient grating signals from ATP-saturated full-length proteins with those from samples completely rid of ATP. Experiment with a non-hydrolyzable ATP analogue (adenylyl imidodiphosphate) should show whether or not the conformational change is triggered by autophosphorylation.

(iii) Finally, the *in-vivo* relevance of the 'STAESSSSGRR---VPE' motif and the proposed HY5-substitution mechanism could be clarified by studying light responses of plants containing CRYs with mutations in this sequence.

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Fig. 1. Proposed mechanism of signal transduction by plant CRY1 (for the example of *AtCRY1*): *I.* Photoexcitation of FAD_{ox}, electron transfer to *FAD from a triad of Trp residues, deprotonation of the terminal Trp by solvent, protonation of FAD⁻/formation of D396⁻. *II.* Reduction of the terminal tryptophanyl radical (stabilization of FADH^{*}, which can no longer recombine with Trp^{*}), D396⁻-induced electrostatic repulsion of ATP resulting in a partial dissociation of CCT from PHR. *III.* Phosphorylation of disclosed serine/threonine residues (within the 'STAESSSS' sequence), formation of a fragment mimicking the sequence of HY5 responsible for binding to COP1. *IV.* Protonation of D396⁻ (or FAD⁻); dissociation of the COP1-SPA1-HY5 complex, substitution of HY5 by CCT at the binding site of COP1, binding of SPA1 by CCT. *V.* Rebinding of ATP by PHR; uncaged HY5 launches transcription of genes implied in the photomorphogenic development, the light signal received by CRY has been transduced. *VI.* Back reoxidation of FADH^{*} to FAD_{ox}, dissociation of COP1 and SPA1 from the CCT. *VII.* Dephosphorylation of phosphorylated acids (controlled by endogenous cellular signals), rebinding of COP1-SPA1 by the PHR. *VIII.* Completion of the signalling cycle, attraction of the positive residues neighbouring the 'STAESSSS' motif on the CCT by phosphates of the PHR-bound ATP, binding of HY5 by the COP1-SPA1 complex, restoring of the dark state of CRY1.

Fig. 2. When partially phosphorylated, the segment of the C-terminal domain of plant CRY1 (bottom; starting with E623 in *AtCRY1*) exhibits a striking structural similarity to the segment of the HY5 transcription factor (top), responsible for its binding to the negative regulator of photomorphogenesis COP1 [27].

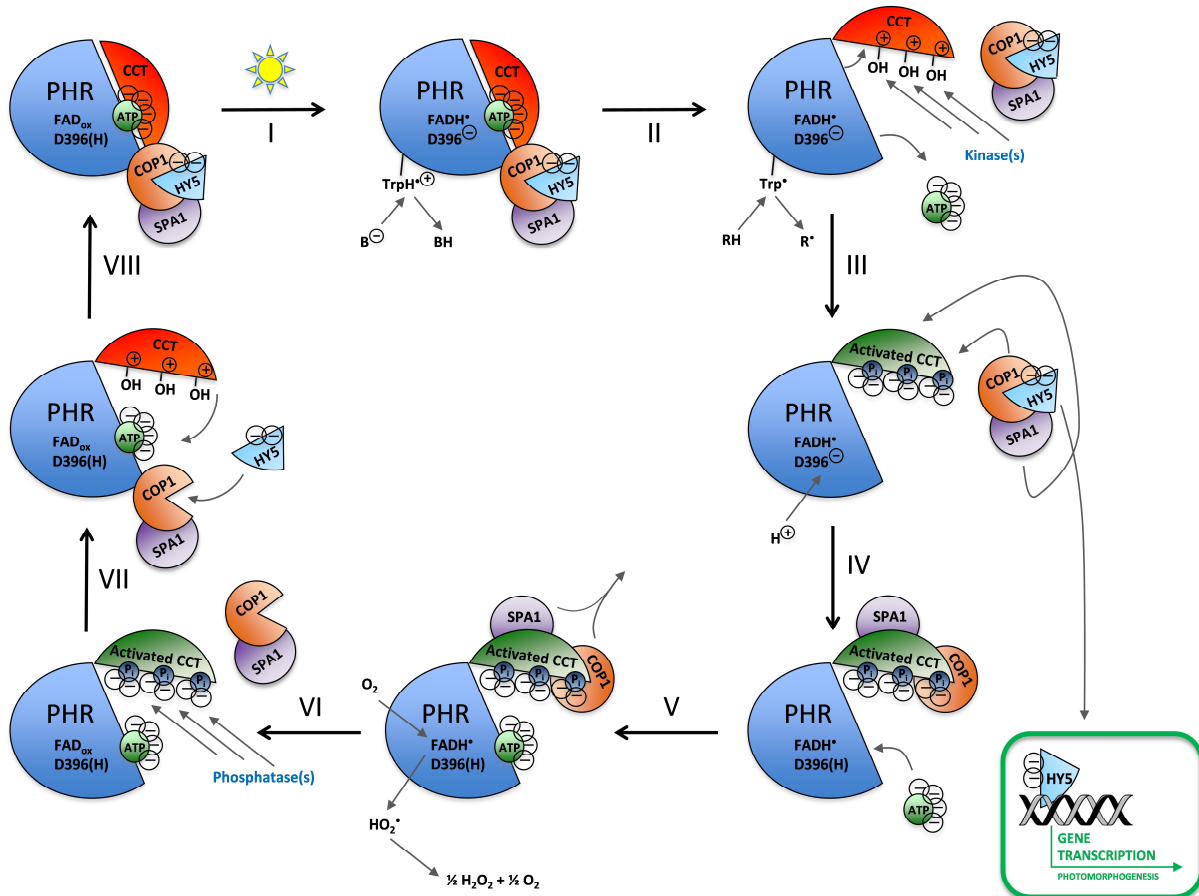
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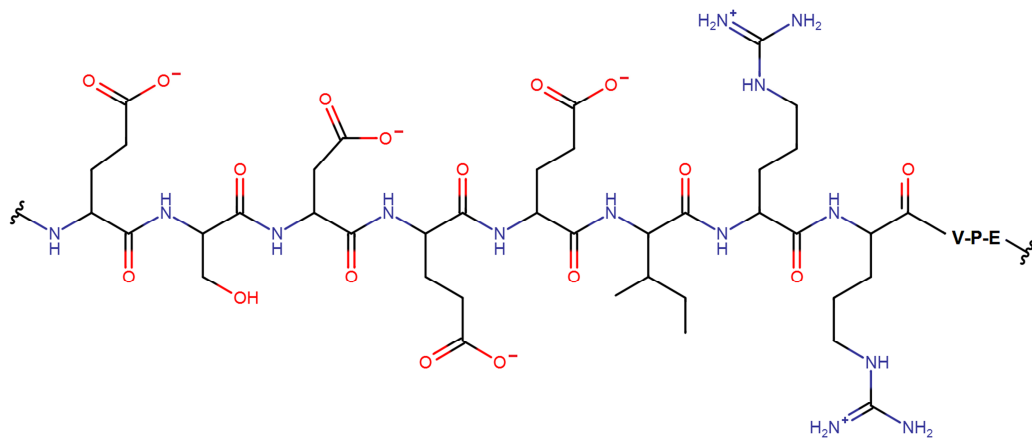
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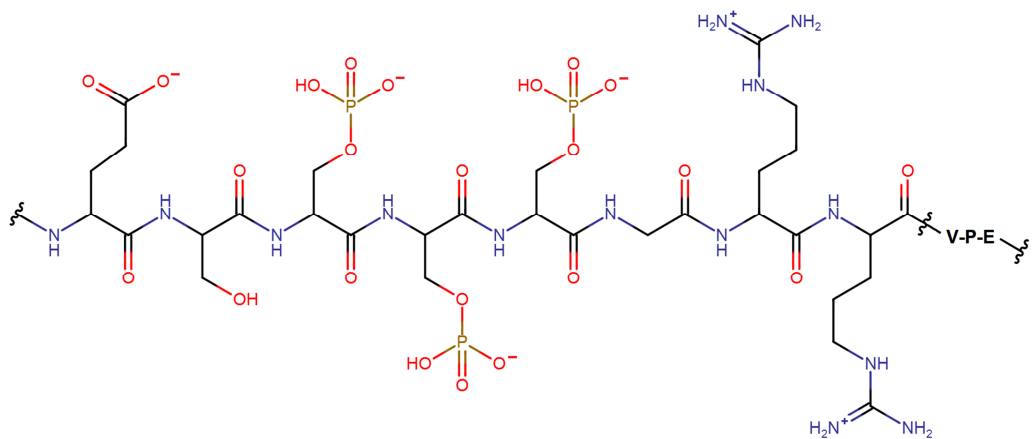
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HY5



Sequence: E S D E E I R R V P E

CCT

Sequence: E S S_p S_p S_p G R R E R S G G I V P E

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Highlights

- We propose a detailed mechanism of signalling by plant photoreceptor cryptochrome1.
- CRY C-terminus is likely activated by a photoinduced negative charge near/on FAD.
- Phosphorylated motif ESSSS...VPE on CRY1 C-terminus resembles a sequence of HY5.
- Photomorphogenesis may be triggered by HY5 substitution on COP1 by CRY C-terminus.

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