LIGHT-INDUCED ELECTRON TRANSFER IN ARABIDOPSIS CRYPTOCHROME-1 CORRELATES WITH IN-VIVO FUNCTION*

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Running title: Function of electron transfer in Arabidopsis cryptochrome-1

Cryptochromes are blue-light activated photoreceptors found in multiple organisms with significant similarity to photolyases, a class of light-dependent DNA repair enzymes. Unlike photolyases, cryptochromes do not repair DNA and instead mediate blue-light dependent developmental, growth, and/or circadian responses by an as-yet unknown mechanism of action. It has recently been shown that Arabidopsis cryptochrome-1 retains photolyase-like photoreduction of its flavin cofactor FAD by intraprotein electron transfer from tryptophan and tyrosine residues. Here we demonstrate that substitution of two conserved tryptophans that are constituents of the flavin-reducing electron transfer chain in E. coli photolyase, impairs light-induced electron transfer in the Arabidopsis cryptochrome-1 photoreceptor in vitro. Furthermore, we show that these substitutions result in marked reduction of light-activated autophosphorylation of cryptochrome-1 in vitro and of its photoreceptor function in vivo, consistent with biological relevance of the electron transfer reaction. These data support the possibility that light-induced flavin reduction via the tryptophan chain is the primary step in the signaling pathway of plant cryptochrome.

Cryptochromes are found in plants, animals, and microbial systems, where they mediate numerous blue-light dependent developmental, growth, and/or circadian responses (1 - 4). Cryptochrome–type photoreceptors are distinguished by their significant similarity to photolyases, a class of DNA repair enzymes (4), that removes lesions in UV-damaged DNA via a light-activated electron transfer mechanism. Despite their similarity to photolyases, and the fact that they bind the same flavin cofactor, FAD, the cryptochromes do not repair DNA and appear to function by interaction with downstream cellular signaling intermediates of the various response pathways (1, 2). The mechanism whereby light activates the cryptochrome photoreceptors, and the significance of their marked structural similarity to photolyases (5-7), is currently unknown.

Photolyases can undergo two distinct light-induced electron transfer reactions upon excitation of their FAD cofactor (4, 8, 9). The first reaction initiates DNA repair and requires the flavin in its fully reduced form. In the second reaction, known as photoactivation, the semi-reduced flavin is converted to the fully reduced form by an electron ultimately provided by an extrinsic reductant. An intraprotein electron transfer pathway connecting the buried flavin to the protein surface has been derived for this photoactivation reaction in E. coli photolyase based on crystallographic structural information and on a combination of site-directed mutagenesis and spectroscopy (10-12). This pathway comprises a chain of three tryptophan residues (W382 - W359 - W306) that are conserved throughout the photolyase/ cryptochrome family. Recently, a study with purified Arabidopsis cryptochrome-1 (cry1) demonstrated occurrence of a similar photoreaction, starting from the fully oxidized form of the flavin and involving tryptophan and tyrosine residues as intrinsic electron donors (13). To explore the possible functional relevance of this reaction to cryptochrome photoreceptor activity, we have substituted redox inactive phenylalanines for two tryptophan residues, W400 and W324, which are found in the cry1 sequence and crystal structure (7) at the homologous positions to W382 and W306 of E. coli photolyase, respectively. The mutant proteins (W400F and W324F) thereby lack the predicted electron donor proximal to the flavin (W400) or exposed to the protein surface...
(W324), and should hence be impaired in electron transfer. If electron transfer is relevant for photoreceptor function, these amino acid substitutions should result in impairment of cry1-dependent blue light responses in vivo.

Experimental Procedures

Preparation of mutant photoreceptor proteins - By means of oligonucleotide-directed mutagenesis, single amino acid substitutions (tryptophan to phenylalanine) were introduced into the coding sequence of cry1 at positions W324 and W400 by using the Altered Sites II in vitro mutagenesis system from Promega (Madison, WI). Mutations were confirmed by sequence analysis. Mutant and wild-type proteins were expressed in a baculovirus expression system and purified by nickel column affinity chromatography as previously described (13,14).

Flavin Photoreduction Assay - Flavin-photoreduction in isolated cry1 was performed by irradiation under anaerobic conditions with white light in the presence of β-mercaptoethanol essentially as described (14). Samples contained between 3 and 24 µM protein, 10 mM β-mercaptoethanol, 0.5 M NaCl, 0.3 M imidazole, 50 mM Tris pH 7.5 and were kept at 12°C during the experiments. Absorption spectra were measured in a Beckmann DU7400 spectrophotometer.

Transient absorption spectroscopy - Transient absorption changes were measured with a time resolution of 100 µs as described (13), using excitation flashes at 355 nm, 5 ns duration, ~5mJ energy per cm², repetition rate 1.983 Hz. Samples contained ~10 µM protein, 0.5 M NaCl and 50 mM Tris pH 7.5 and were kept at 12°C during the experiments. 32 signals were averaged for each sample.

Autophosphorylation assay - Isolated chromochrome was radiolabeled as described (15). 2 µg protein were incubated with [γ-32P]ATP for 10 minutes at 22°C, either in darkness or under broad band blue light (400 – 500 nm, 30 µmoles m⁻² s⁻¹), resolved on polyacrylamide gels and stained with coomassie dye to visualize the protein bands. Gels were subsequently dried and subjected to autoradiography.

Phenotypic analysis of Arabidopsis mutant seedlings - Mutant cry1 coding sequences were cloned behind the strong 35S promoter of the binary vector pKYLX6 and transformed into cryptochrome-deficient (cry1cry2 mutant) Arabidopsis plants via agrobacterium-mediated transformation as described (16). Arabidopsis seedlings expressing wild-type or mutant cry1 were analyzed after 5 days of growth in continuous blue light (30 µmoles m⁻² s⁻¹) according to methods and procedures described and/or referenced (16,17). Anthocyanin accumulation was quantified by extraction of pigments from 30 seedlings in 1 ml of acidified methanol and measurement of the absorbance at 530 nm, corrected for the contribution of chlorophyll as described (16,17).

Results

Wild type and mutant cry1 proteins expressed in a baculovirus expression system were checked for pigment content by steady state absorption spectroscopy. As previously reported for wild type cry1 (14), both W324F and W400F mutant proteins bound the FAD cofactor in fully oxidized form as evidenced by its characteristic absorption spectrum in the 400 to 500 nm region (Fig.1).

We applied two different methods to determine whether the mutations affected light-induced electron transfer in the cry1 photoreceptor in vitro, (i) a global assay of flavin photoreduction (Fig. 1), and (ii) a time-resolved study of intraprotein electron transfer (Fig. 2). In the photoreduction assay, wild type and mutant proteins were subjected to continuous illumination in the presence of the reductant β-mercaptoethanol, and flavin reduction was monitored by absorption changes in the 400-600 nm region. Wild type protein showed progressive flavin reduction during the course of a half-hour illumination as previously described (14), very little reduction was seen either in the W400F or W324F mutant protein (Fig.1 inset), consistent with interruption of the electron transfer pathway needed for flavin photoreduction.

As a specific test for the occurrence of intraprotein electron transfer, photoreactions were studied in the absence of external reductant on a much more rapid time scale. The fully oxidized flavin cofactor was excited by a short laser flash and the resulting photoreactions were monitored by transient absorption spectroscopy with 100 µs time resolution. As shown previously (13), the rapid absorption increase at 520 nm in wild type cry1 (Fig. 2, upper trace) reflects concomitant formation of the semireduced neutral flavin radical and of a neutral tryptophanyl radical due to tryptophan-
to-flavin electron transfer. The subsequent polyphasic absorption decay has been attributed to reduction of the tryptophanyl radical by a tyrosine residue, and to electron backtransfer from the flavin radical to the tryptophanyl and tyrosyl radicals. For both the W324F and W400F mutant proteins, we observed absorbance changes that were approximately five times weaker than in the wild type protein (Fig. 2), strongly suggesting an important role of these two tryptophan residues for flavin photoreduction.

During the transient absorption studies, we noticed a strong enhancement in flash-induced fluorescence for the W400F mutant protein (visible in Fig. 2 as a negative spike). According to separate fluorescence measurements under identical excitation conditions, the fluorescence yield ratios were approximately 8:2:1 for W400F, W324F and wild type proteins, respectively (not shown). The strongly enhanced fluorescence in the W400F mutant protein suggests that W400 is the primary electron donor to the flavin in wild type cry1, and that this nearby tryptophan quenches fluorescence of the fully oxidized flavin due to fast electron transfer, as suggested for other flavoproteins (19,20).

To determine a possible functional role for cry1 of the light-induced electron transfer, we examined the effect of the same amino acid substitutions on an in vitro autophosphorylation reaction found in purified preparations of cry1 (15,21). This autophosphorylation activity is both light and redox-sensitive, requires the presence of the flavin cofactor within the apoprotein, and has been proposed as an early step in light activation of the photoreceptor (15). Both W324F and W400F mutant proteins showed basal levels of phosphorylation in the dark, reduced in comparison to wild type protein at the same concentration (Fig.3). Significantly, there was no increase in autophosphorylation of mutant protein samples due to illumination by blue light as compared to the dark controls. This was in marked contrast to the wild type protein, where significant stimulation by blue light occurred.

To explore the functional relevance of the electron transfer reaction in vivo (Fig.4), W324F and W400F mutated coding sequences were cloned into a plant expression vector and were transformed into cryptochrome-deficient Arabidopsis plants. Transgenic seedlings were evaluated by Western blot analysis with anti-cry1 antibody to verify synthesis of the mutant photoreceptor proteins (Fig.4b). As functional assays for cry1 – specific activity in vivo, transgenic seedlings were evaluated for blue-light dependent inhibition of hypocotyl elongation (Fig.4a,d) and for blue-light dependent anthocyanin accumulation (16,17) (Fig.4c), in comparison to seedlings containing wild type cry1 protein at a similar concentration. By both these functional assays, activity was shown to be significantly reduced in the two transgenic lines containing a mutant cry1 photoreceptor (W324F and W400F) as compared to seedlings containing wild type cry1 (Fig.4c,d).

Discussion

The present study documents a strong correlation between a primary photochemical reaction (electron transfer) and cry1 photoreceptor function, both in vitro, and in vivo. We show that substitution of tryptophan residues that are homologous to those that participate in the photoactivation reaction of E. coli photolyase result in marked impairment in electron transfer of isolated cry1 photoreceptors. This impairment is unlikely to be caused by major structural perturbations, as the efficient FAD binding observed in the mutants implies proper contacts with multiple amino acid residues throughout the protein (7). Furthermore, amino acid substitutions at the homologous positions in E. coli photolyase do not impair DNA repair activity (18). Hence, although we cannot exclude the possibility of minor structural alterations in the protein resulting from these mutations, we attribute the impairment in electron transfer in the mutants to most likely result from the elimination of essential members of the electron transfer chain. Our results thus suggest that functional electron transfer along the triple tryptophan chain is conserved between plant cryptochromes and their photolyase homologues, revealing a functional significance of the marked amino acid similarity between cryptochromes and photolyases. Although greatly reduced, there was residual electron transfer in the mutant proteins (Fig.2). This may be due to low yield side pathways involving other tryptophan and/or tyrosine residues that are close to the flavin. According to the crystal structure of the photolyase-like domain of wild-type cry1 (7), best candidates are W385 (at 7.1 Å edge-to-edge distance from the flavin), W356 (7.1 Å), W334 (9.2 Å), W377 (9.7 Å) and Y402 (6.9 Å). For
comparison, W400 is at 4.5 Å from the flavin. Based on an excited state lifetime of several nanoseconds for the fully oxidized flavin (22) and on the distance dependence of intraprotein electron transfer rates (23), oxidizable residues within ~10 Å could well reduce the photoexcited flavin with non-negligible yields.

A light-dependent autophosphorylation reaction has been speculated to be an early step in activation of the cryptochrome photoreceptor (15, 21). In our in vitro phosphorylation assay (Fig.3), both the W400F and W324F mutant proteins retained basal (light–independent) autophosphorylation, indicating that they retain the capacity for undergoing the autophosphorylation reaction, although somewhat reduced in comparison to wild type protein. The prominent stimulation of autophosphorylation by blue light observed in wild type was, however, completely suppressed in both the W400F and W324F mutant proteins, indicating that intraprotein electron transfer is necessary for light stimulation of this autophosphorylation response.

In vivo photoreceptor function of cryptochrome was markedly reduced in both substitutions (W324F and W400F), as measured both by reduced anthocyanin accumulation and reduced hypocotyl growth inhibition under blue light in transgenic plants (Fig. 4). We detected some residual activity in the W324F and W400F mutants. This activity may have resulted from residual electron transfer activity via alternate (and less efficient) electron transfer pathways, as discussed above. Summarizing, our data suggest that light-induced electron transfer provides the trigger for light responsivity of the cry1 photoreceptor.

In photolyases, photoactivation converts the semi-reduced flavin with absorption bands in the 500-650 nm region to the fully reduced form that can participate in the DNA repair reaction and has no pronounced absorption bands in the visible. This photoactivation reaction is normally not essential for function as photolyases are typically found in the fully reduced form in living cells and proteins deficient in photoactivation can still function in repair (4,18). In the case of plant cryptochromes, action spectroscopy has indicated that there is no significant response to wavelengths of light above 500 nm and that the response maximum is around 450 nm, suggestive of flavin in the oxidized form in vivo (24). Therefore, a primary light reaction for cryptochrome whereby the oxidized flavin undergoes light-dependent photoreduction would appear a reasonable mechanism, that may then trigger subsequent conformational or other biochemical changes that have been suggested to be the basis for signaling (25).

Cryptochrome-like photoreceptors have been identified in a number of other organisms including animals, where they have been shown to play an important role in the circadian clock (1-4). There are significant structural similarities between animal and plant cryptochromes, including conservation of the three tryptophan residues that have been implicated in the electron transfer chain of E. coli photolyase and which we have here demonstrated to be implicated in light-dependent electron transfer in plant cry1. Substitution of some of these tryptophan residues by tyrosine has been reported to reduce activity of Xenopus cryptochromes in an in vitro transfection assay (26). However, since the effect of cryptochrome on reporter gene expression is independent of light in the transfection assay, it can not be concluded from this experiment that electron transfer is involved in animal cryptochrome activity. On the other hand, mutations of the homologous tryptophans to phenylalanines in Drosophila cryptochrome (27) did not abolish its light-dependent activation either in transfection assays or at the level of protein stability. Therefore it would appear unlikely, in Drosophila cryptochromes, that light activation depends on electron transfer via the same pathway functioning in the plant cry1. Since plant and animal cryptochromes apparently evolved independently from different photolyase ancestors (4), they may have a different primary mechanism of action. Alternatively, electron transfer in animal-type cryptochromes may still be important for light activation, but may occur via an alternative pathway to that found in plant cryptochrome. Indeed, the type 6-4 photolyases to which the animal cryptochromes are most closely related may utilize an electron transfer pathway involving primarily tyrosine (28). It is therefore intriguing to speculate that the electron transfer activity present in photolyases contains latent signaling potential that has been co-opted for a role in blue-light photoreceptors multiple times throughout the course of evolution.
REFERENCES


FOOTNOTES

* M.A. acknowledges support by CNRS

1 Abbreviation used: cry1 – *Arabidopsis* cryptochrome-1
FIGURE LEGENDS

Fig. 1. Flavin-photoreduction in isolated wild type as well as W324F and W400F mutant cry1 proteins under continuous illumination. Absorption spectra were taken before illumination (“dark” spectra; normalized for comparability) and after various illumination times as indicated (spectra only shown for wild type; mutant spectra changed very little upon illumination (see inset) and were omitted for clarity). The absorption bands in the 400 to 500 nm region that decreased upon illumination result from fully oxidized flavin, while the absorption above 500 nm that was induced by the illumination results from the neutral semi-reduced flavin (29). Inset, decrease in absorbance at 447 nm due to reduction of the flavin cofactor as a function of illumination time for wild-type (triangles) and for W324F (squares) and W400F (circles) mutant proteins.

Fig. 2. Laser flash-induced electron transfer in isolated wild-type (WT) as well as W400F and W324F mutant cry1 proteins. Transient absorption changes were measured at 520 nm. Amplitudes were rescaled to compensate for slightly different cry1 concentrations of the three samples. The mutant signals are vertically and horizontally offset for clarity.

Fig. 3. Light-induced autophosphorylation of isolated wild type as well as W324F and W400F mutant cry1 proteins. ‘Dark’ and ‘Light’ indicate radiolabeling in the dark and under blue light, respectively.

Fig. 4. Functional analysis of W324F and W400F cry1 mutants in vivo. WTcry1, wild type seedlings; WTcry1*, seedlings overexpressing wild-type cry1 in a cryptochrome–deficient (cry1cry2 mutant) background; — , seedlings expressing no cryptochromes (cry1cry2 mutant); W324Fcry1 or W400Fcry1, seedlings expressing the respective mutant cry1 in a cryptochrome-deficient (cry1cry2) background. a Visible phenotype. b Western blot analysis of levels of cry1 protein expressed in seedlings as shown in panel a. c Anthocyanin accumulation (mean + s.d. from three independent experiments). d Hypocotyl growth inhibition. Hypocotyl length of thirty seedlings was measured for each point (mean + s.d.).
Fig. 1 (M. Ahmad)
Absorbance change at 520 nm ($10^{-4}$) vs. Time (ms)

- WT
- W400F
- W324F

Fig 2 (M. Ahmad)
Fig. 3 M. Ahmad
Figure 4 (M. Ahmad)
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