Limited solvation of an electron donating tryptophan stabilizes a photoinduced charge-separated state in plant (6–4) photolyase

Yuhei Hosokawa, Pavel Müller, Hirotaka Kitoh-Nishioka, Shigenori Iwai & Junpei Yamamoto

(6–4) Photolyases ((6–4) PLs) are ubiquitous photoenzymes that use the energy of sunlight to catalyze the repair of carcinogenic UV-induced DNA lesions, pyrimidine(6–4)pyrimidone photoproducts. To repair DNA, (6–4) PLs must first undergo so-called photoactivation, in which their excited flavin adenine dinucleotide (FAD) cofactor is reduced in one or two steps to catalytically active FADH− via a chain of three or four conserved tryptophan residues, transiently forming FAD−/FADH−⋯TrpH•+. Photolyases and related photoreceptors cryptochromes use a plethora of tricks to prevent charge recombination of photoinduced donor–acceptor pairs, such as chain branching and elongation, rapid deprotonation of TrpH•+ or protonation of FAD−. Here, we address Arabidopsis thaliana (6–4) PL (At64) photoactivation by combining molecular biology, in vivo survival assays, static and time-resolved spectroscopy and computational methods. We conclude that At64 photoactivation is astonishingly efficient compared to related proteins—due to two factors: exceptionally low losses of photoinduced radical pairs through ultrafast recombination and prevention of solvent access to the terminal Trp3H•+, which significantly extends its lifetime. We propose that a highly conserved histidine residue adjacent to the 3rd Trp plays a key role in Trp3H•+ stabilization.

Photolyases and cryptochromes constitute a superfamily (PCSf) of ubiquitous structurally related photoactive flavoproteins. The evolutionarily older photolyases (PLs) have specialized in the photoenzymatic repair of major UV-induced lesions: cyclobutane pyrimidine dimers (CPDs; specifically repaired by CPD PLs) and pyrimidine(6–4)pyrimidone photoproducts (6–4)PPs; specifically repaired by (6–4)PLs). Cryptochromes (CRYs), which branched off from photolyases, have gradually lost their ability to repair DNA, but have progressively acquired new physiological functions as blue light receptors driving photomorphogenesis in plants or entraining the circadian clock in both plants and animals. Numerous theoretical and experimental works suggest that animal cryptochromes, which group together with animal (6–4) photolyases, are also responsible for the ability of migratory birds and other animals to sense the Earth's magnetic field and use it for orientation.

PCSf proteins undergo two distinct kinds of photoreactions: photorepair and photoactivation. In photorepair, which is unique to photolyases and a few rare ‘dual’ proteins (capable of acting as both DNA repair enzymes and photoreceptors), the photoexcited fully reduced flavin (‘FADH−’) transfers an electron to the DNA lesion fixed in a nearby specific binding pocket. This electron transfer (ET) triggers bond rearrangement within the lesion, which, upon electron return to the intrinsically semi-oxidized (or semi-reduced) FADH−, ultimately leads to restoration of two intact bases and thus to DNA repair.

Nevertheless, the flavin cofactor in PLs is not always in the catalytically active (fully reduced) form and therefore needs to be activated (reduced). This happens in the latter reaction called photoactivation. Photoactivation, which is common to both PLs and CRYs, is a light-induced reduction of the oxidized (FADox) or semi-reduced (FADH•) flavin chromophore via a chain of electron-transferring aromatic residues, typically three tryptophans. Upon excitation, ‘FADox’ or ‘FADH•’ abstract an electron from a nearby Trp residue (TrpH), producing a ~4 Å charge-separated state (FAD−/FADH−⋯TrpH•+). TrpH•+ subsequently gets an electron from yet another Trp residue, and the resulting radical cation TrpH•+ in turn acquires an electron from yet another Trp residue.
residue near the protein surface\textsuperscript{10–12} (Trp\textsubscript{3H}), as illustrated in Fig. 1a. The successive ET on the sub-nanosecond time scales yields a ~15 Å charge-separated state (FAD\textsuperscript{•−}/FADH\textsuperscript{−}···Trp\textsubscript{3H}\textsuperscript{•+}). Finally, deprotonation\textsuperscript{13} of Trp\textsubscript{3H}\textsuperscript{•+} to Trp\textsubscript{3}\textsuperscript{•} and quenching of Trp\textsubscript{3}\textsuperscript{•} by external reducing agents\textsuperscript{14} stabilize the FAD\textsuperscript{•−}/FADH\textsuperscript{−} state\textsuperscript{15} (see Fig. 1b detailing the first photoactivation step starting with FAD\textsubscript{ox} and yielding the semi-reduced FAD\textsuperscript{•−}/FADH\textsuperscript{•}). While the catalytically active redox form of FAD in DNA repair by PLs is FADH\textsuperscript{−}, light signaling by CRYs is believed to be triggered by FAD\textsubscript{ox} photoreduction to FAD\textsuperscript{•−} (or by a negative charge on a neighboring residue that protonated FAD\textsuperscript{•−} to FADH\textsuperscript{•})\textsuperscript{16–18}. Most CRYs hence seem to use only the first photoactivation step.

To ensure efficient photoactivation, PCS\textsubscript{f} proteins employ a plethora of ingenious tricks stabilizing the light-induced radicals and preventing futile charge recombination: e.g., branching and/or elongation of the ET chain to four or even five residues\textsuperscript{21–23}, rapid (~μs) protonation of FAD\textsuperscript{•−} (unique to plant cryptochromes)\textsuperscript{24}, or rapid (sub-ns) deprotonation of the last member of the ET chain. The latter can be achieved by the presence of a proton acceptor next to the terminal member of the ET chain (e.g., a deprotonated aspartic or glutamic acid\textsuperscript{25} or a cluster of structured water molecules communicating with the surrounding buffer\textsuperscript{26}) or by using tyrosine as the terminal residue of the chain\textsuperscript{25,26} (while Trp\textsubscript{H•+} has a pK\textsubscript{a} of ~4\textsuperscript{27}, Tyr\textsubscript{H•+} has a pKa of ~−2\textsuperscript{28} and deprotonates immediately in aqueous buffers).

In this study, we focused on the (6–4) photolyase from Arabidopsis thaliana (At\textsubscript{64}), which, despite possessing a mere Trp triad, exhibits surprisingly high activity in bacterial cells—comparable to that of Xenopus laevis (6–4) PL (Xl\textsubscript{64})\textsuperscript{29}, which contains a Trp tetrad, and the photoactivation of which is known to yield very stable charge separation and unusually long-lived radical pairs\textsuperscript{31}. Our results suggest that At\textsubscript{64} achieves efficient photoactivation by using a different set of tricks than other PCS\textsubscript{f} proteins: 1) rapid localization of the electron hole on the 3rd Trp (which we infer from an unusually high (>80%) quantum yield of the FAD\textsuperscript{•−} Trp\textsubscript{H•+} pairs, implying proportionally low losses of primary and secondary FAD\textsuperscript{•−} Trp\textsubscript{H•+} pairs by ultrafast recombination), and 2) prevention of solvent access to Trp\textsubscript{H•+}, which extends its lifetime by a factor of ten (compared to a mutant with solvent-exposed 3rd Trp). We propose that a histidine residue adjacent to the 3rd Trp and highly conserved in

Figure 1. Scheme of FAD photoreduction in PCS\textsubscript{f} proteins. (a) The crystal structure of At\textsubscript{64} (PDB entry 3FY4)\textsuperscript{19} is superimposed onto a homology model structure of Xl\textsubscript{64} using the crystal structure of (6–4) PL from Drosophila melanogaster (6–4) PL (PDB entry 3CVU)\textsuperscript{20} as template. FAD is shown in yellow, electron-transferring tryptophans and redox-inactive phenylalanine in At\textsubscript{64} and Xl\textsubscript{64} are shown in green and in salmon, respectively. (b) Reaction scheme detailing typical photo-induced charge transfer, charge separation and radical stabilization in PCS\textsubscript{f} proteins with Trp triads and tetrads. Trp\textsubscript{H}, Trp\textsubscript{H•+}, and Trp\textsuperscript{•} denote the normal (non-oxidized) tryptophan state, the one-electron-oxidized cation radical, and the one-electron-oxidized deprotonated neutral radical, respectively.
plant (6–4) PLs plays a key role in stabilization of TrpH⁺, thus expanding the arsenal of tricks leading to efficient photoactivation of PCSf proteins.

Results

Primary and tertiary structures reveal major differences in the environment of TrpH in plant and animal (6–4) PLs. To address how plant (6–4) PLs are finely adapted to FAD photoreduction via the Trp triad, we assumed that residues around TrpH affect the formation and/or stabilization of the photoinduced FAD⁻ TrpH⁺ charge-separated state. To explore the differences in the environment of TrpH in plant and animal (6–4) PLs, the crystal structure of At6419 (PDB entry 3FY4) was superimposed onto a homology model structure of Xl64 generated as previously described2. (Fig. 2a). Apart from the phenylalanine residue (Phe380 in At64), which is replaced by the fourth tryptophan in animal (6–4) PLs (Trp370 in Xl64), the only major difference in the closest neighborhood of TrpH (within 4 Å) is that a histidine residue conserved in plant (6–4) PLs (His382 in At64) is substituted by a serine in a serine (6–4) PLs (Ser372 in Xl64). The remaining residues around TrpH are either conserved (Trp383 and Met318 in At64 numbering) or very similar (Ile327 in At64 vs. Val317 in Xl64).

Our sequence analyses revealed that as many as 95% of compared plant (6–4) PL orthologues retain the His residue, and 92% of the animal (6–4) PL orthologues retain the Ser residue (Figs. 2b,c). Interestingly, in no plant (6–4) PL orthologues is the histidine replaced by a serine, suggesting that the histidine residue could play a functional or at least an auxiliary role in the stabilization of TrpH⁺ and thereby in the photoreduction of plant (6–4) PLs.

Mutation of His382 to Ser in At64 slows down the photoreduction in vitro and impairs the photorepair capability in bacterial cells. To examine whether the His/Ser difference affects FAD photoreduction via the Trp-triad, we investigated steady-state photoreduction kinetics for the H382S mutant of At64 (At64-H382S), as previously performed for the wild-type of At64 (At64-WT). At64-H382S was illuminated by >430 nm light in the presence of an external reductant under anaerobic conditions and the UV/Vis absorption spectral changes were monitored. Upon light illumination, the absorption band at 450 nm characteristic of FADH⁻ (via transient accumulation of small amounts of FADH⁺, as indicated by the absorption growth and decay between 500 and 700 nm; Supplementary Fig. 1). Fitting the normalized absorption at 450 nm (A₄₅₀) with a monoexponential decay function revealed that FAD photoreduction in At64-H382S occurred with a half-life (t₁/₂) of 222 ± 4 s, which is about tenfold slower than that in At64-WT (t₁/₂ = 21.6 ± 0.5 s) under the same conditions (Fig. 3a). To examine whether the less efficient photoreduction caused by the H382S mutation affects the (6–4)PP photorepair activity of At64, UV-sensitive E. coli SY32 cells, in which CPD lesions can be repaired due to a rescue plasmid coding for trpH (pSY32), were transformed with an At64-H382S expressing plasmid, in the same way as previously reported. The survival rate was found to be about tenfold lower in H382S than in WT (Fig. 3b). These results indicate that the H382S mutation in At64 impairs the (6–4)PP photorepair in bacterial cells by hampering the preceding (and necessary) photoreduction reaction.

In the previous study on FAD photoreduction in Xl64 containing a Trp-tetrad, the mutation of TrpH in Xl64 to Phe (Xl64-W370F) showed a comparatively slow photoreduction kinetics under the same conditions (Fig. 3c, t₁/₂ = 297 ± 19 s). Interestingly, the W370F/S372H double mutant of Xl64 (Xl64-W370F/S372H), which mimics the circumstance of the Trp-triad in At64, exhibited ~ fourfold faster (t₁/₂ = 73 ± 4 s) photoreduction kinetics than Xl64-W370F (Fig. 3c), illustrating that the introduction of the His residue elevated the photoreduction capability.
replacement of His382 by Ser, Asn, and Asp residues resulted in an increased solvation of Trp3H compared to molecules in HS2 in each frame. As expected, the water molecule distribution in HS2 demonstrated that the through HS2 and HS2 could be susceptible to the mutation of His382, we first counted the number of water and the second (HS2) hydration shell, respectively (Fig. 5a). Because bulk water molecules could come into HS1 in the course of the simulation time, while the mutants could have more than two molecules in a frame. We recently reported that a water molecule was stably captured proximal to Trp3H during a MD runs. We defined the area within 3.4 and 5.0 Å of the nitrogen atom of the Trp3H indole ring as the first (HS1) (Fig. 5b, and Supplementary Table 1). To examine the influence of the solvation in Photoreduction experiments on other His382 mutants. In general, His residues play a variety of roles in biological functions based on their polarity, acid-base properties, and the planarity/aromaticity. To identify which feature of His382 in At64 controls the photoreduction via the Trp-triad, various His382 mutants were subjected to the photoreduction experiment. Although some mutants could not be isolated (presumably due to the loss of the structural integrity), we successfully purified the H382D, H382N, H382V and H382Y mutants of At64 without any apparent structural or functional perturbations as confirmed by their typical spectral changes during the photoreduction process (Supplementary Fig. 2). Their photoreduction kinetics were found to be 4.3, 3.3, 2.4, or 1.6-fold slower than At64-WT for the H382D, H382N, H382V and H382Y mutant, respectively (t_{1/2} = 92 ± 5 s for H382D, t_{1/2} = 71 ± 4 s for H382N, t_{1/2} = 53 ± 5 s for H382V, t_{1/2} = 34 ± 3 s for H382Y, Fig. 4).

The slowest photoreduction kinetics of H382D among the tested mutants indicates that the capacity of His382 to act as a proton acceptor for Trp3H\(^{(+)}\) is likely not required to improve the photoreduction efficiency. The faster kinetics of H382V vs. that of H382N indicates that increased polarity near Trp3H is not beneficial either. The most similar kinetics to At64-WT was observed for the H382Y mutant. This result suggests the possibility that the planarity/aromaticity or just plain bulkiness of the His and Tyr residues might be the key factor that boosts the photoreduction efficiency. Interestingly, the His → Tyr substitution is found in (6–4) PL orthologues from primitive plants such as Lycopodiopsida and Bryophyta (our Blast-p search showed that the His residue is conserved among 95% of plant (6–4) PL orthologues and the rest has a Tyr residue in this position, Fig. 2b).

Altogether, the photoreduction experiments on the His382 mutants suggest that small and polar residues are not good alternatives to His382 and that the proton-accepting ability and/or polarity of His382 are not required to make the FAD photoreduction in At64 efficient.

Molecular dynamics simulations suggest that the regulated solvation of Trp3H could play an essential role in the photoreduction of At64. Given the observations that FAD photoreduction in At64 significantly slowed down upon mutation of His382 to relatively compact and polar residues (Ser, Asn, and Asp), we considered the possible involvement of solvation of Trp3H in the photoreduction. To evaluate the solvation, we performed molecular dynamics (MD) simulations for the His382 mutants in the same way as previously reported, and analyzed the presence of water molecules around the Trp3H in the last 100 ns of the production runs. We defined the area within 3.4 and 5.0 Å of the nitrogen atom of the Trp3H indole ring as the first (HS1) and the second (HS2) hydration shell, respectively (Fig. 5a). Because bulk water molecules could come into HS1 through HS2 and HS2 could be susceptible to the mutation of His382, we first counted the number of water molecules in HS2 in each frame. As expected, the water molecule distribution in HS2 demonstrated that the replacement of His382 by Ser, Asn, and Asp residues resulted in an increased solvation of Trp3H compared to Val, Tyr, and His residues (Fig. 5b, and Supplementary Table 1). To examine the influence of the solvation in HS2 on that in HS1, we also analyzed the water molecule distribution in HS1 (Supplementary Fig. 3), showing a clear difference between the WT protein and all the His mutants. Indeed, WT exclusively bore only one water molecule in HS1 in the course of the simulation time, while the mutants could have more than two molecules in a frame. We recently reported that a water molecule was stably captured proximal to Trp3H during a MD simulation.
simulation for WT (Wat1 in Fig. 5a), and that the mutation of Ser412 (hydrogen-bonding to Wat1) to Ala significantly reduced the photoreducibility of At64. As Wat1 is located in HS1, the observed single water molecule in the water molecule distribution in HS1 is assigned to be Wat1. It is conceivable that the mutation of His382 alters the coordination of Wat1, however, as shown in Supplementary Table 2, the distance between the oxygen atom of Wat1 and the nitrogen atom of the indole ring of Trp3H (dO…N) during 100 ns simulation time for all the mutants was approximately the same as in WT. This result suggests that the mutation of His382 does not significantly affect the Wat1 coordination and that another structural aspect is likely engaged in the fine-tuning effect of His382.

Thus, we hypothesized that the presence and/or behavior of the additional water molecules (other than Wat1) in HS1 could affect the photoreduction in the His382 mutants of At64. To explore the dynamic behavior of water molecules, we counted the total numbers of the water molecules coming in and out of the HS1 during the 100 ns simulation, by tracing the ID of the water molecules (Fig. 5c and Supplementary Table 3). The results clearly show that there is a tendency that mutants with better water accessibility to HS1 are more difficult to photoreduce. As described above, Wat1 was the only water present in HS1 of WT, suggesting that His382 would play a role in preventing water access to Trp3D. Interestingly, the result for the H382Y mutant, which exhibited similar photoreduction kinetics to WT (Fig. 4), indicated that only one additional water molecule (Wat2) came into and out of HS1, and that other randomly moving water molecules outside HS1 did not enter HS1. Noteworthy, Wat2 in H382Y has been captured at the position corresponding to the place occupied by His382 in WT during the simulation time (Supplementary Fig. 4). The limited access of random water molecules into HS1 in H382Y (Fig. 5c) suggested that Wat2 prevents other water molecules from approaching Trp3H, in a similar way as His382 in WT. In summary, our MD simulation and photoreduction experiments indicate that a more solvent-accessible environment around Trp3H in the H382S, H382N, and H382D mutants could have a negative impact on their photoreduction, while the photoreduction of At64-WT and H382Y seems to be enhanced by regulating the solvent access to Trp3H.

According to the Marcus theory describing ET reactions, solvation of the reaction partners can affect the free energy difference (ΔG), the reorganization energy (λ), and the ET rate. We thus estimated the effect of His382 on ΔG and λ for the ET from Trp3H to Trp3H+ by classical MD simulations for WT and H382S (see Supplementary Methods). However, the calculated ΔG and λ values for H382S differed only slightly from those obtained for WT (Supplementary Table 4), suggesting that His382 does not significantly affect these parameters.

Transient absorption spectroscopy on ns-µs timescales reveals that the regulated solvent accessibility impedes Trp3H+ deprotonation in At64-WT. In order to further clarify the effects of mutations on the fate of the photoinduced FAD"-Trp3H+ radical pair in At64, we compared WT with the least photoreducible mutant H382S by transient absorption spectroscopy in the ns-to-µs regime (Figs. 6a,b), where Trp3H+ deprotonation and/or FAD"-Trp3H+/Trp1 charge recombinations are typically observed in PCF proteins. Based on the reference absorption spectra of the expected photoinduced species (Fig. 6c), we decided to follow the photoreaction at two selected wavelengths: at 457 nm, which is close to the maximum of the expected bleaching of the FADox absorption band (due to its reduction to FAD") and where the Trp radicals do not contribute much to the absorption change; and at 562 nm, which is close to one of the two maxima of the TrpH+ absorption band and where the absorption changes due to FADox reduction to FAD" (and subsequent reoxidation upon radical pair recombination) are expected to be relatively small.

Figure 4. FAD photoreduction kinetics for H382Y (red), H382V (orange), H382N (green), and H382D (blue) At64 mutants reflected by the decay of normalized A450 values compared to WT (grey). All normalized A450 plots could be reasonably fitted by monoexponential decay functions. Spectral data for the mutants are available in Supplementary Fig. 2.
Upon excitation by ~5 ns flashes at 480 nm, bleaching at 457 nm and absorption increase at 562 nm with the same initial ratio of the two signals of ~ −1.5:1 (consistent with the formation of FAD•− TrpH•+ radical pairs) in both protein samples were induced, followed by a decay of all signals to zero within less than 50 µs (Figs. 6a,b), suggesting that all photoinduced radicals recombined in this time window. However, the signal decays were markedly different in the two proteins: while the decay of the WT signals at both wavelengths was essentially monoexponential with a time constant τ1 of ~1 µs (according to the fit, traces of a slower τ2 ~ 8 µs process amounting to less than 10% of the signal amplitudes were present), the decay of the H382S signals was clearly biexponential, with τ1 of ~100 ns and τ2 of ~9.5 µs. To qualitatively describe the processes occurring with these kinetics, we analyzed the amplitude ratio of the signals at 457 and 562 nm (|ΔA457/ΔA562|; Fig. 6d). This ratio remains constant in the case of a simple radical pair recombination, but it is sensitive to changes in the chemical nature of the radicals. TrpH•+ cation radical absorbs significantly more than the neutral Trp• radical at 562 nm (see Fig. 6c), and TrpH•+ deprotonation hence leads to absorption decrease at this wavelength and to a corresponding increase in the |ΔA457/ΔA562| value. In the case of At64-WT, the amplitude ratio (Fig. 6d) changes only very little, indicating that the major process behind the ~1 µs decay of signals in Fig. 6a is charge recombination of the initially produced FAD•− TrpH•+ pairs. The deprotonation of TrpH•+ radicals is apparently very slow in At64-WT and can hence compete with FAD•− TrpH•+ recombination only to a very limited extent. On the other hand, the amplitude ratio in the H382S mutant dramatically increases within the first 300 ns (with the time constant of ~100 ns), indicating that TrpH•+ deprotonation occurring within this kinetic phase is much faster in H382S (Fig. 6d). After the initial growth phase, the |ΔA457/ΔA562| value stabilizes at ~2.4. The following decay of the transient absorption signals in ~9.5 µs (Fig. 6b) hence reflects pure recombination of the FAD•− Trp• pairs.

Analysis of the phase amplitudes obtained from the biexponential fits of the signals from At64-WT confirms that >90% of the FAD•− TrpH•+ radical pairs directly recombine within the ~1 µs kinetic phase (the amplitudes of this phase are in a good agreement with the difference spectrum of FAD•− – FADox + TrpH•+; see Supplementary Fig. 5a). The remaining <10% of pairs, in which tryptophan cation radicals do deprotonate, decay in ~8 µs. Indeed, the amplitudes of this minor phase fit well the difference spectrum of FAD•− – FADox + Trp• (Supplementary Fig. 5a). In At64-H382S, the situation is markedly different. Since the TrpH•+ cation radicals rapidly deprotonate within the first ~100 ns kinetic phase (the amplitudes of this phase are consistent with the TrpH•+ – Trp• difference spectrum, see Supplementary Fig. 5b), only a very small fraction of FAD•− TrpH•+ pairs recombine directly (in competition with the fast deprotonation). The fraction of the remaining FAD•− Trp• pairs (Supplementary Fig. 5b) is hence much larger in H382S than in the WT protein. Nevertheless, the lifetimes of the FAD•− Trp• pairs are comparable in both proteins (~8 µs in WT vs. ~9.5 µs in H382S).

Given that the ~1 µs FAD•− TrpH•+ decay phase in WT reflects to ~90% recombination and to ~10% TrpH•+ deprotonation, the intrinsic time constant for TrpH•+ deprotonation is ~10 µs in this protein. This means that while the H382S mutation has virtually no impact on the lifetime of the FAD•− Trp• pairs (see above), it accelerates the TrpH•+ deprotonation by a factor of ~100 (from ~10 µs in WT to ~100 ns in H382S) and shortens the lifetime of the FAD•− TrpH•+ pairs by a factor of ~10 (from ~1 µs in WT to ~100 ns in H382S). To our knowledge, TrpH•+...
deprotonation in At64 in ~ 10 µs is the slowest terminal TrpH•+ deprotonation rate ever reported for a WT PCSf protein (the second-slowest being the Trp4H•+ deprotonation in Xl64 in ~ 2.5 µs under very similar conditions21). When estimating the energy of the excitation pulses using the [Ru(bpy)3]2+ actinometer37, we also estimated the quantum yield (see SI) of the photoinduced FAD•− TrpH•+ pairs detected at ‘time zero’ of our experiment, i.e., pairs that had not been lost due to ultrafast recombination processes faster than our time resolution (i.e., ~ 5 ns, limited by the excitation pulse length). The yield of the detected FAD•− TrpH•+ radicals is ~ 80% in both At64-WT and -H382S, which means that the losses due to ultrafast FAD•− TrpH•+ recombination are only ~ 20%. The similar quantum yield of stable FAD•− Trp3H•+ pairs in At64-WT and -H382S indicates that His382 does not have a significant impact on the electron transfer through the Trp triad in At64.

Discussion
Upon photoactivation, A. thaliana (6–4) photolyase exhibits by far the slowest (~ 10 µs) deprotonation of the terminal TrpH•+ cation radical of all PCSf proteins studied to date. In the absence of extrinsic reductants in vitro, the consequence of this slow deprotonation rate is that most (~ 90%) of the photoinduced FAD•− TrpH•+ radical pairs recombine as such (in ~ 1 µs) and the remaining ~ 10% of FAD•− Trp• pairs (in which Trp3H•+ has been deprotonated) recombine in ~ 8 µs. Putting the ~ 10 × faster FAD photoreduction in At64-WT (than in H382S) in vitro (Fig. 3a) and the ~ tenfold higher survival rate of WT-expressing E. coli cells in the repair activity assay (compared to cells expressing At64-H382S; Fig. 3b) into context with these results, it seems that the extrinsic reductants act much more easily upon TrpH•+ than upon Trp•, both in vitro and in vivo. This is likely because TrpH•+ reduction requires a mere ET, while Trp• reduction requires transfer of not just an electron but also of a proton (or of a hydrogen atom H•) and because ET can occur over longer distances than the transfer of a proton.
or of a hydrogen atom. In any case, formation of a much greater fraction (compared to WT) of the longer-lived \( \text{FAD}^{•−} \) \( \text{Trp}^{•} \) pairs in H382S does not seem to be able to compensate to any visible extent for the ~10 × shortened lifetime of the \( \text{FAD}^{•−} \) \( \text{TrpH}^{+} \) pairs in this mutant. The slow deprotonation of the terminal tryptophan cation radical and the consequently extended lifetime of the \( \text{FAD}^{•−} \) \( \text{Trp3H}^{+} \) pair (and likely also that of the \( \text{FADH}^{−} \) \( \text{Trp3H}^{+} \) pair in the second phot activation step) considerably enhance the yield of \( \text{At}^{64} \) photoactivation (and hence the rate of \( \text{FAD} \) photoreduction under continuous illumination)—by providing the extrinsic reductants more time and opportunity to encounter and quench \( \text{Trp3H}^{+} \) at the protein surface and thereby stabilize the reduced flavin in the protein interior by preventing recombination with its radical counterpart.

Searching for the cause of the unusually slow \( \text{Trp3H}^{+} \) deprotonation, we identified a neighboring histidine residue, which is highly conserved in plant \((6–4) \text{PLs (His382 in} \text{At}^{64} \text{numbering), and the mutation of which (to diverse alternative residues—Tyr, Val, Asn, Asp and/or Ser) indeed had a significant negative effect on the rate of} \text{At}^{64} \text{photoactivation in vitro. In the case of the in vivo-tested (and the least in vitro-photoactivatable)} \text{H382S mutant, the mutation also had a dramatically deleterious impact on the survival of UV-irradiated and Vis-reactivated} \text{E. coli} \text{cells, suggesting that the His382 residue next to the terminal (3rd) tryptophan likely plays an important role also in the in vivo photoactivation of} \text{At}^{64} \text{via the Trp chain.}

According to our MD simulations, the native His382 blocks access of solvent molecules to the nitrogen atom of \( \text{Trp}^{•+} \) \( \text{Trp}^{•−} \) in \( \text{At}^{64} \) (Fig. 7), and it does so much more efficiently than any alternative amino acid in the tested mutants. In the WT protein, the only water molecule interacting with \( \text{Trp3H}^{+} \), referred to as \( \text{WAT1} \) in this text, is tightly coordinated by other amino acids within \( \text{At}^{64} \). It never leaves the first hydration shell of the nitrogen atom of \( \text{Trp3H}^{+} \) and it does not interact with the bulk buffer enough to provide a functional channel for a proton transfer from \( \text{Trp3H}^{+} \) to the buffer.

Finally, the efficiency of \( \text{At}^{64} \) photoactivation is further enhanced by exceptionally low losses (~20%) due to ultrafast (<5 ns) recombination of the photoinduced \( \text{FAD}^{•−} \) \( \text{Trp}^{•−} \) and/or \( \text{FAD}^{•−} \) \( \text{Trp2H}^{+} \) radical pairs. This is significantly less than in any other PCSf protein studied so far. For comparison, in \( \text{E. coli} \) CPD PL ~35% of the photoinduced radical pairs are lost within the first few ns48, in \( \text{Chlamydomonas reinhardtii} \) animal-like CRY it is ~50%49, in \( \text{Xl}^{64} \) ~75%45, and in AtCRY 80 to 95%, depending on the presence or absence of ATP17. In \( \text{Dinoroseobacter shibae} \) NewPHL, which is an ancestral PL containing a mere Trp dyad, as much as ~90% of the photoinduced radical pairs are lost within the first few nanoseconds and most of the remaining ~10% pairs recombine within the next ~50 ns29. Interestingly, our structural comparison between \( \text{At}^{64} \) and \( \text{Xl}^{64} \) does not show any remarkable difference in the residues within 4 Å of \( \text{FAD} \) and \( \text{Trp}^{•} \) (Supplementary Fig. 6a), but the residues around \( \text{Trp3H} \) are different. Notably, an arginine residue (Arg387) is located near \( \text{Trp3H} \) in \( \text{At}^{64} \), which is replaced by glutamine (Gln377) in \( \text{Xl}^{64} \) (Supplementary Fig. 6a). This arginine is highly conserved in plant \((6–4) \text{PLs, but is not found in any animal (6–4) PL (Supplementary Figs. 6b,c). The positively-charged guanidinium moiety of Arg387 could destabilize the localization of the hole on \( \text{Trp3H} \) and accelerate the successive electron transfer from \( \text{Trp3H} \), leading to the ultrafast charge separation between \( \text{FAD} \) and \( \text{Trp3H} \) and low recombination losses. This hypothesis will be addressed by future experimental and computational studies.
Methods

Plasmid construction. For the (6–4)PP repair activity assay in bacterial cells, and protein production, pGEX-4T-1 and pET28a (+) plasmids that produce the (6–4) PLs were constructed, respectively. First, the desired mutations (W329F and H382S) of At64 were introduced into the pGEX-4T-1 plasmid carrying the At64-WT gene with the QuickChange Site-Directed Mutagenesis Kit. The PCR primers used for the mutagenesis are shown in Supplementary Table 5 (Entry 4 and 5). After sequencing, the obtained plasmids were used for the assay.

The mutant genes in the pGEX plasmids were then amplified and subcloned into the NdeI/Xhol restriction site of the pET-28a (+) vector with the specific primers as shown in Supplementary Table 5 (Entry 8). The H382D, H382N, and H382V mutations of At64 were also introduced into the pET28a vector carrying the At64-H382S gene by the QuickChange Site-Directed Mutagenesis kit (Supplementary Table 5, Entry 2). As for the H382Y mutant, the 5' upstream and 3' downstream of the mutation site were separately amplified using the primers shown in Supplementary Table 5 (Entry 6), and the amplicons were fused into the pET-28a (+) vector linearized with the Ndel and Xhol treatment, by using the In-fusion HD Cloning Kit (Takara). The S372H mutation of Xl64 was introduced into the pET28a (+) vector carrying the reported Xl64-W370F gene using the In-fusion HD Cloning kit with the specific primers shown in Supplementary Table 5 (Entry 7). The obtained plasmids were sequenced and used for the protein production.

(6–4)PP repair activity assay in bacterial cells. The E. coli SY32 strain (uvrA−, recA−, phr+), in which CPD PL activity is rescued by the pACYC184 plasmid coding E. coli CPD PL gene, has been used for (6–4) PP repair activity assay in bacterial cells. SY32 cells were transformed with a pGEX-4T-1 plasmid expressing a (6–4) PL variant and the colonies were selected on Luria broth (LB) agar plates containing tetracycline (10 µg mL−1) and ampicillin (80 µg mL−1). The transformant was cultivated in a 1.5 mL of LB medium containing tetracycline and ampicillin at 37 °C overnight. The culture was diluted to OD600 = 0.5 with an LB medium. The diluted culture was induced with 2.4 µL of 10 mg mL−1 isopropyl-β-D-thiogalactoside (IPTG) and shaken at 37 °C for 1 h. The culture was appropriately diluted with phosphate-buffered saline, and 150 µL of aliquots were spread onto LB agar plates containing tetracycline and ampicillin. The plates were irradiated with 20 W UV germicidal lamp (UVL20PH-6, Sen Lights Co Ltd., Osaka, Japan) through metal mesh filters (2.0 µW cm−2) calibrated with a 254 nm probe, UVP LLC, Upland, CA) for 15 or 30 s to yield a total irradiance of 0.3 or 0.6 J m−2, respectively. The plates were subsequently illuminated with fluorescent lamps (18 W × 4, FL20SSD/18, Toshiba, Tokyo, Japan) for 30 min, and then incubated at 37 ºC overnight. After incubation, the number of colonies was counted taking into account the dilution percentage. All survival rates were normalized to the number of colonies obtained without UV irradiation. The experiments were independently performed in triplicate (n = 3), and the statistical significance was analyzed with a Student's t-test. The significant cutoff value was set to 0.05.

Protein purification. For purification of At64 variants, we modified a previously reported expression and purification protocol. E. coli C41 (DE3)/pLysS (Lucigen) cells were transformed with a pET-28a (+) plasmid encoding the (6–4) PLs gene and grown in a 2 L of LB medium containing kanamycin (20 µg mL−1) in a 5 L flask with baffles at 37 °C. When OD600 reached 1.2, the culture was cooled to 25 °C. Protein production was then induced with a final concentration of 0.2 mM IPTG, and the culture was further incubated at 25 °C for 24 h. After harvest, the pellet was frozen with liquid nitrogen and thawed on ice. The cells were resuspended in 40 mL of a lysis buffer (20 mM Na2HPO4, 500 mM NaCl, 5% glycerol, pH 7.4 adjusted by KOH, plus 65 mg of lysozyme) and lysed by sonication. The cell lysate was centrifuged, and the supernatant was loaded onto a HiTrap Heparin HP column (GE Healthcare) with an elution buffer (20 mM NaH2PO4, 500 mM NaCl, 500 mM imidazole, 5% glycerol, pH 7.4 adjusted by KOH). The His-tagged protein was eluted by increasing the NaCl concentration to 500 mM with an elution buffer (20 mM NaH2PO4, 500 mM NaCl, 10 mM imidazole, 5% glycerol, pH 7.4 adjusted by KOH), and the His-tagged protein was eluted with an elution buffer (20 mM NaH2PO4, 500 mM NaCl, 500 mM imidazole, 5% glycerol, pH 7.4 adjusted by KOH). For further purification, the green eluate was loaded onto a HiTrap Heparin HP column (GE Healthcare) and purified with a step-gradient of 100–500 mM NaCl in a buffer containing 50 mM Tris–HCl and 5% glycerol (pH 8.0). The purified protein was confirmed by 10% SDS-PAGE, and its concentration was measured based on a FAD absorbance at 450 nm using a molar extinction coefficient of 11.300 L mol−1 cm−1.

Measurement of steady-state photoreduction kinetics by UV–Vis absorption spectroscopy. Steady-state photoreduction of recombinantly produced proteins was performed under anaerobic conditions as previously described. 70 µL of protein diluted to 20 µM was applied to a Micro Bio-Spin 6 column (BIO-RAD) equilibrated with a reaction buffer (20 mM phosphate, 500 mM NaCl, 10% glycerol, pH 7.5). An aliquot (60 µL) of the eluate was transferred into an anaerobic 10 × 2 × 8 mm (length × width × height) inner volume quartz cuvette (Starna, 16.160-F/4/Q/10 GL 14/2/Z15). After the cuvette was sealed with a screw cap and PTFE-coated silicone and rubber septa, the air inside the cuvette was replaced with nitrogen through the septa. Further preparation was performed in an anaerobic glovebox. The sample was mixed with L-cysteine (the final concentration was 5 mM) and the reaction buffer up to a total volume of 240 µL under anaerobic and dark conditions.

The anaerobic samples were illuminated with continuous light (430–800 nm) from a MAX-150 xenon lamp (Asahi Spectra) through the 10 mm × 8 mm window on ice (illumination time varied depending on the sample). After shaking the sample gently, an absorption spectrum was recorded through the 10 mm path by a UV–Vis spectrometer Lambda 35 UV–Vis spectrometer (PerkinElmer) or V-730 spectrometer (JASCO). The absorbance
at 450 nm of the obtained spectra was plotted and fitted with a monophasic exponential decay function with the Origin2019 software.

**Transient absorption spectroscopy.** The proteins were dissolved in a buffer consisting of 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 5% glycerol, at final concentrations of ~125 µM for At64-WT and ~95 µM for At64-H382S. The transient absorption setup has been described in detail elsewhere. The laser energy was estimated from transient absorption signals using [Ru(bpy)₃]²⁺ as an actinometer. Monitoring light at two selected wavelengths (457 and 562 nm) was provided by continuous-wave lasers (Cobolt Twist™ and Oxxius 561–25-COL-002, respectively). The measuring light was perpendicular to the excitation beam and passed through the sample along the 10-mm path of a 2 x 2 x 10 mm (W x H x L) quartz cell with self-masking solid black walls (Starna). Flash-induced changes of the transmission of the sample were monitored behind the sample by a Si photodiode (Alphalas UPD-500-UP, < 500 ps rise time) coupled to a Tektronix MSO 64 digital oscilloscope with bandwidth limit set to 200 MHz. All shown traces are averages of 16 signals recorded with a repetition rate of 2 Hz. The samples were measured at room temperature. Transient absorption kinetics were determined using the Levenberg–Marquardt least-squares optimization algorithm in Origin 2020 (by OriginLab), globally fitting the trend lines according to the equation: \( y(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + \gamma_0 \).

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Author contributions

Y. H., S. I., and J. Y. conceived the idea of the work; Y. H. performed the survival assay, protein production, and photoreduction experiments; Y. H. and H. K.-N. conducted MD simulations; H. K.-N. calculated the electron transfer parameters; P. M. carried out the transient absorption spectroscopic experiments; Y. H., P. M., H. K.-N., and J. Y. wrote the manuscript; All authors have analyzed the data, reviewed and given approval to the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to J.Y.

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