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ARTICLE

Photoinduced transformation of UVR8 monitored by vibrational and fluorescence spectroscopy

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Tryptophan residues at the dimer interface of the plant photoreceptor UVR8 promote monomerisation after UV-B absorption via a so far unknown mechanism. Using FTIR spectroscopy we assign light-induced structural transitions of UVR8 mainly to amino acid side chains without major transformations of the secondary structure of the physiologically relevant C-terminal extension. Additionally, we assign the monomerisation associated increase and red shift of the UVR8 tryptophan emission to a photoinduced rearrangement of tryptophan side chains and a relocation of the aspartic acid residues D96 and D107, respectively. By illumination dependent emission spectroscopy we furthermore determined the quantum yield of photoinduced monomerisation to 20 +/- 8 %.

Introduction

In contrast to classical photoreceptor proteins that employ light-absorbing pigments to perceive light, the UV-B (280 – 315 nm) responsive protein UVR8 from *Arabidopsis thaliana* uses tryptophan side chains as a proteinogenic chromophore.[1, 2] The dimeric form in the absence of UV-B contains a delicately assembled cross-dimer salt-bridge network between the β-propeller structures of the monomers and harbours the photoreceptive tryptophans arranged in an excitonically coupled cross-dimer pyramid (W94, W233, W285, W337).[3, 4] Upon UV-B illumination the protein monomerises both *in vivo* and *in vitro*.[5] The salt-bridge network maintaining the dimeric form in the dark consists of aspartates, glutamates and arginine residues. In particular, the residues R286, D96 and D107 are crucial for dimer formation.[3, 4] The UVR8 monomer is the signalling active form *in vivo* and UV-B absorbance leads to a rapid nuclear accumulation of UVR8 as well as a directed interaction of UVR8 with COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1) via the C-terminal extension of UVR8 (C27).[6-9] Besides UV-B induced monomerisation the molecular details of photoactivation of UVR8 are unknown. It was hypothesized that either cation-π interactions between the pyramid tryptophans and neighbouring salt-bridge arginines are disturbed by illumination or that neutralization of the arginine side chain by light-induced electron transfer (ET) from tryptophan takes place.[3, 4] Both processes are expected to induce a disruption of the cross-dimer salt-bridge interaction. Recent computational studies support the ET mechanism leading to a neutralization of the cross-dimer salt-bridges by coupled electron and proton transfer.[10] Another recent work postulates W285/W233 ET resulting in a charge separated state (W^+/W^-) that may disturb the dimer interaction by its large dipole moment.[11] Moreover, charge relocation in the excited state of W285 has been proposed to destabilize nearby salt bridges.[12] Experimental evidence for either mechanism has yet to be found. In a recent time resolved fluorescence spectroscopy study, however, ultrafast quenching of the terminal W285 residue and resonance energy transfer from distal tryptophans has been observed.[13] The latter may indicate a light-harvesting role for the remaining tryptophans in UVR8.
Here, we studied UVR8 WT and selected key mutants by vibrational and fluorescence spectroscopy. With these methods sensitive to structural and chemical changes of the whole protein and selectively for the electronic nature of the chromophore we obtained important insights into the photoactivation of UVR8 and their implications for signalling.

Experimental

Protein preparation

UVR8 protein samples were expressed and purified as previously described.[3] For H/D exchange a lyophilized protein sample was dissolved in D_2O and incubated for >72 hours. C-

terminal truncation between R406 and Y407 was carried out by trypsin digestion as previously described.[3] The complete cleavage of the C-terminus was confirmed by SDS-PAGE (not shown).

FTIR difference spectroscopy

Light-minus-dark difference spectroscopy was carried out using a Bruker IFS66/s spectrometer as previously described with a resolution of 4 cm^{-1}.[14] Protein concentrations were adjusted to an OD_{280nm} between 60-80/cm and about 5-10 µL were sandwiched between two CaF_2 plates without spacers and greased for tightness. Illumination was carried out using an LED (UV LED-280, Laser2000 GmbH, Wessling, Germany), with an emission centred at 280 +/- 15 nm (FWHM) and an output power of ~0.5 mW. The spectra present an average of 100 scans of light-minus-dark difference spectra corrected with the corresponding number of scans of dark-minus-dark spectra.

Fluorescence spectroscopy

Steady state fluorescence measurements were carried out using a Jobin Ivon HORIBA Fluorolog Tau-3 lifetime system. UVR8 samples were diluted in a total volume of 2 ml to an OD_{280nm} ~0.05 /cm in a 1x1 cm quartz cuvette and their emission was recorded from 285 nm to 501 nm using excitation at 280 nm. For the time dependent behaviour of the emission spectra the sample was stirred and the spectrometer was set to an excitation slit of 1 nm and an emission slit of 5 nm. The acquisition time per spectrum with a resolution of 2 nm was 38 s. The power of the excitation beam was 40 +/- 10 µW (Newport 1918-R power meter / Newport 818-UV/DB detector). Emission spectra have been corrected for Raman scattering of the aqueous medium by subtracting a Gaussian at 313 nm. Global analysis was performed using the Glotaran software package.[15]

Results and discussion

Light-induced structural changes of UVR8 observed by FTIR difference spectroscopy

So far molecular structural information is neither available for the monomeric active form of the protein nor the C27 domain, which is necessary for the physiologically crucial COP1 interaction.[7] Using FTIR difference spectroscopy on isolated UVR8 protein we revealed structural transitions of full length UVR8 upon UV-B illumination. Care was taken that the samples were well hydrated. Difference spectra were only obtained for WT samples and site-directed mutants of UVR8 that were able to undergo dimer to monomer transitions. The observed spectral signatures therefore correspond to the structural properties of dimer (negative contributions) and monomer (positive contributions). The light-minus-dark difference spectrum of functional UVR8 in H_2O (Figure 1A) features prominent difference signals between 1750 and 1300 cm^{-1}, with the strongest signals at 1686 (-), 1634 (+) and 1517 (-) cm^{-1}. Surprisingly the solvent exchange of H_2O to D_2O resulted in only minor spectral shifting, which indicates that the overall secondary structure of UVR8 remains largely unchanged upon monomerisation (Figure 1B). It should be noted though, that although the samples have been incubated for more than 72 hours in D_2O, H/D exchange is not necessarily complete. In this case the sample was previously illuminated with UV-B in D_2O, which due to the long lifetime of the monomeric state *in vitro* would lead to a pronounced exposure to D_2O of structural elements at the dimer interface (Figure S1). The H/D insensitivity of the spectra implies that the overall secondary structure elements of UVR8 are very rigid. The largest D_2O induced difference is found in the amide I frequency range between 1600 and 1700 cm^{-1}, where the 1651(+) cm^{-1} signal completely disappears upon H/D exchange. This frequency range may correspond to amide I vibrations associated with α-helical structures. UVR8 contains very short α-helical elements, which are found within the connecting loops of the individual propeller blades and the C-terminal extension. Interestingly, one such partly helically structured connector is situated at the dimer interface and harbours the salt-bridge forming D96 (Figure 5A), that is expected to undergo structural changes upon monomerisation. However, the D96N mutant shows a highly similar light-induced difference spectrum and therefore rules out this assignment (Figure 2). Alternatively, the frequency is also characteristic for the guanidino groups of arginine side chains. The frequency of the strongest C-N stretching vibrations in guanidino compounds is strongly influenced by the dipolar environment and very susceptible to H/D exchange, which may lead to deuterium induced downshifts of up to 50 cm^{-1}.[16] In salt bridges formed with strongly H-bonding counter-ions like carboxylic acids, the frequency is expected to shift up to 1690 cm^{-1}.[17] Due to their insensitivity to H/D exchange the prominent difference signals at 1686(-), 1673(+), 1666(+), 1634(+) cm^{-1} are very unlikely to correspond to arginine residues.[16] Given the large number of water molecules at the interface a shielding effect for these residues is unlikely.[18] Secondary structure changes related to turns may be reflected by the large negative feature at 1686 cm^{-1} and the low frequency component of the 1673/1666 cm^{-1} double peak that is slightly downshifted by 2 and 3 cm^{-1}, respectively.

Another prominent change upon H/D exchange is the reduced amplitude of the negative signal at 1517 cm^{-1} and an increase in the bleach between 1550 and 1525 cm^{-1}. Reduced difference signal amplitudes can arise from changed extinction coefficients, signal width and by the shift of an underlying or closely neighbouring signal. The former has been observed for tyrosine in solution around this frequency, where the exchange of the phenolic OH group to OD resulted in an increase in the extinction coefficient of the C-C ring vibrations without any strong spectral shift.[16]

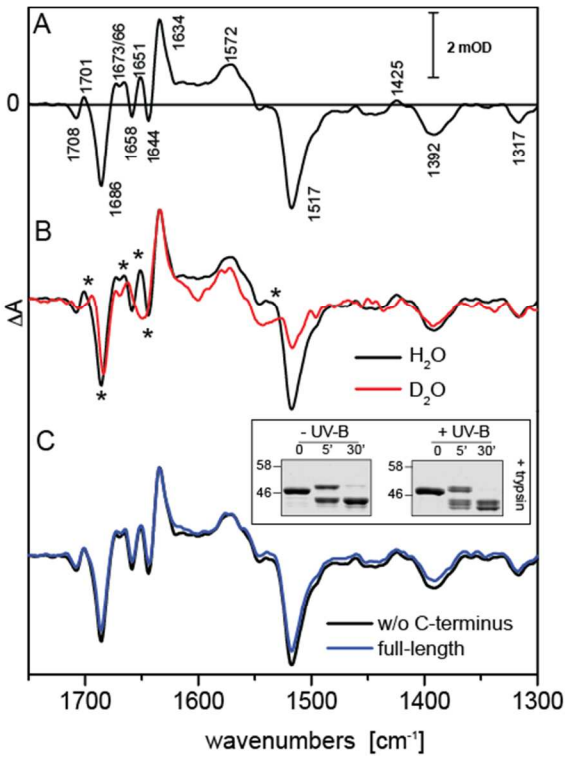

Figure 1. Light-minus-Dark FTIR difference spectra of UVR8 WT (A). H/D induced spectral changes of UVR8 WT are indicated by an asterisk (B). Truncation of the C-terminal domain does not affect the difference spectrum of UVR8 WT (C). The inset illustrates the increased susceptibility of the C-terminus to trypsin digestion upon UV-B illumination.

In UVR8 light-induced secondary structural changes are also expected for the physiologically relevant C27 domain, which interacts with COP1 in a light-dependent manner.[7] We therefore compared FTIR spectra of the full-length protein with that of a C-terminally truncated version (Figure 1C). The light-minus-dark difference spectra are virtually identical, which rules out any conformational changes in the deleted region of the C-terminus. Nevertheless, in darkness C27 appears to be shielded from interaction with COP1.[3, 4, 7] Light-induced exposure of C27 without any conformational switching is therefore a conceivable signalling mechanism. This possibility is further supported by the increased susceptibility of the C-terminus to proteolytic cleavage of the protein upon monomerisation *in vitro* (inset Figure 1C) and the fact that an antibody that recognises the extreme C-terminus binds selectively to UVR8 in plant extracts only after UV-B exposure.[5] A crystal structure of the full-length protein including the C-terminal region is unfortunately not available so far, probably due to a high conformational flexibility in this region. A structural model derived from solution SAXS experiments points to a distal location of the C-terminal extensions away from the dimer interface,[1] which renders the un-caging model for C27 less conceivable unless there are additional requirements for the interaction with COP1 situated at or close to the dimer interface. Furthermore, additional proteins may be necessary to facilitate a conformational change in the C-terminus. Possible candidates are the COP1 protein itself or RUP (REPRESSOR OF UV-B PHOTOMORPHOGENESIS) proteins, which have been shown to promote re-dimerization *in vivo*.[19]

Light-induced difference signals outside of the typical amide I region can be tentatively assigned to specific amino acid side chain functionalities according to their typical frequencies. The most likely corresponding weakly positive and negative features at 1708(-) and 1701(+) cm^{-1} are characteristic for carbonyl stretching modes of carboxylic acids like aspartate or glutamate side chains. Usually this mode is not observed in deprotonated carboxylate groups, since the carboxylate anion is resonance stabilized and both C-O bonds have equivalent bond orders. In UVR8 D96 and D107 form salt bridges to R286 according to the crystal structures (Figure 5), which in combination are essential for dimerization.[3] The asymmetric nature especially of the D96 salt bridge may account for a changed bond order in the carboxylate groups, rendering one C-O bond to have more double bond character and thus giving rise to a weak carbonyl-like vibration. Additionally, further cross-dimer salt bridges (E182/R146 and D44/R338) are expected to contribute to these FTIR signals. It cannot be ruled out completely though that other aspartate and glutamate residues within the protein core are represented by this signal as well. The observed frequency in the dark state indicates H-bonding[16] and the putative H/D sensitive downshift of this vibration in the light state suggests a stronger coordination in the monomer. In line with this hypothesis the signal is affected by the D96N or D107N mutation (Figure 2).[3] The D96N and/or D107N mutant additionally feature differences in the broad 1572(+) and the 1392(-) cm^{-1} signal, which is characteristic for the asymmetric and respectively symmetric stretching vibration of side chain carboxylate groups. The positive and respective negative sign of the signals indicate that in the transition from dimer to monomer the symmetric vibrations, probably arising from the strong bi-dentate coordination in the salt bridge, lose intensity in favour of a more asymmetric, mono-dentate coordination of the carboxylate group. Indeed the crystal structure of the UVR8 dimer shows that D107 is in direct bi-dentate coordination of R286 (Figure 5A).[3] D96 in contrast features only one direct interaction with R286, but is involved in an H-bond network trough a water molecule to Y253 and W285 (not shown).[3] Accordingly, the more downshifted signal at ~1390 cm^{-1} signal in the mutant lacking D107 may reflect the different coordination state of D96.

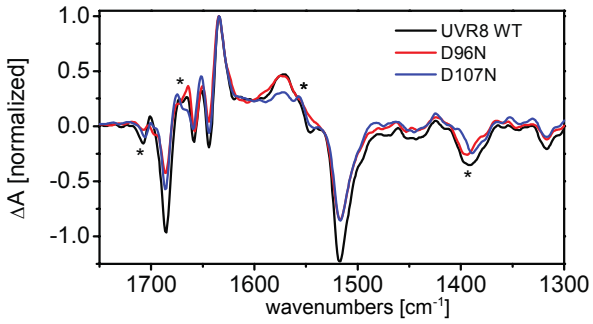

Figure 2. Light-minus-Dark FTIR Difference spectra of UVR8 WT (black) and the mutants D96N (red) and D107N (blue).

Another major change in the transition from dimer to monomer is the disruption of the tryptophan pyramid, which results in a different environment for these residues. The difference spectra of UVR8 feature signatures at 1517(-) and the weak 1425(+) cm^{-1} that are characteristic for tryptophan indole side chains.[16] Additional aromatic ring vibrations of tryptophan and tyrosine may occur at around 1620 cm^{-1}. At 1517 cm^{-1} also contributions from phenol groups of tyrosine should be considered, especially due to the observed H/D induced change in signal amplitude as described above. The role of tyrosines at the dimer interface in the photoactivation of UVR8, however, is still completely unclear. As they are clearly involved in the hydrogen bond network on the monomer surface surrounding the tryptophan pyramid, structural changes are expected as well for these residues. In a time resolved resonance Raman study on bacteriorhodopsin a downshift of the so-called W3 vibration at ~1550 cm^{-1} was assigned to an increase in steric repulsion of a tryptophan side chain due to its susceptibility to the dihedral angle between the indole ring and the Cβ atom.[20] Although tryptophan signals in this region are expected to be weak in IR spectra,[16] the signal here might still be attributed to a reorientation of indole rings in the monomer. However, due to the various molecular contributions in the infrared absorption at these frequencies observed for indole,[16] this could also simply reflect a change in hydrogen bonding and/or dipolar interaction between the pyramid tryptophans.

Photoinduced emission changes of UVR8

The electronic nature of the tryptophan residues can be addressed by fluorescence spectroscopy. The properties of the tryptophan emission are direct indicators of the environment of these residues.[21] Here, we recorded illumination dependent emission spectra of UVR8 and mutants. Upon monomerisation the peak emission of the bulk tryptophan residues red shifts from 327 nm to 335 nm along with a 3-6 fold increase in fluorescence intensity (Figure 3). According to a thorough statistical analysis on the microenvironments of tryptophan residues in a great number of proteins by Reshetnyak and coworkers[21] these emission wavelengths are typical for class I tryptophans, but the 327 nm emission of the dark-adapted state may also be attributed to class S residues. Class I tryptophans differ from class S residues in a lower packing density (number of surrounding atoms) and higher accessibility of the Nε1 and Cζ2 atoms, which usually orient to polar environments. This correlation could indicate a loosening of the packing and increase of solvent accessibility of the tryptophan residues at the interface upon monomerisation. The total solvent accessibility is still low for class I residues and they are still considered buried. Partially solvent exposed tryptophan side chains (class II) have been shown to emit at ~340 nm. A clear interpretation here however is extremely difficult due to the overlap of protein buried and interfacial tryptophan emission observed in the bulk fluorescence here. Moreover, the closely clustered and excitonically coupled interfacial tryptophan residues are expected to have different electronic properties than the isolated tryptophan residues analyzed by Reshetnyak and coworkers[21].

Upon further illumination the fluorescence decreases again along with a slight broadening of the overall emission spectrum to red.[4] Since the emission of the constitutively monomeric R286A mutant only features a minor shift from 337 nm to 339 nm upon illumination (Figure 4), the red shift is considered to be an indicator for the light-induced monomerisation. Global analysis of the WT data using a model of sequentially interconverting species ($1 \Rightarrow 2 \Rightarrow 3$) determined three significant kinetic components with their corresponding evolution associated spectra (EAS) to describe the change of the emission properties under constant illumination. The time constant of 738 s is assigned to the formation of the red shifted state with increased intensity (red EAS) from the dark-state (black EAS) for these illumination conditions (Figure 3). Subsequently, by relating the number of monomer molecules at a given time to the total number of absorbed photons by the dimer we determined a quantum yield of 20 +/- 8% for the photoinduced dissociation of UVR8. It should be noted that this parameter drastically depends on the number of tryptophan side chains actually participating in the photoactivation and can easily be about twice higher if only the interfacial tryptophans are considered.

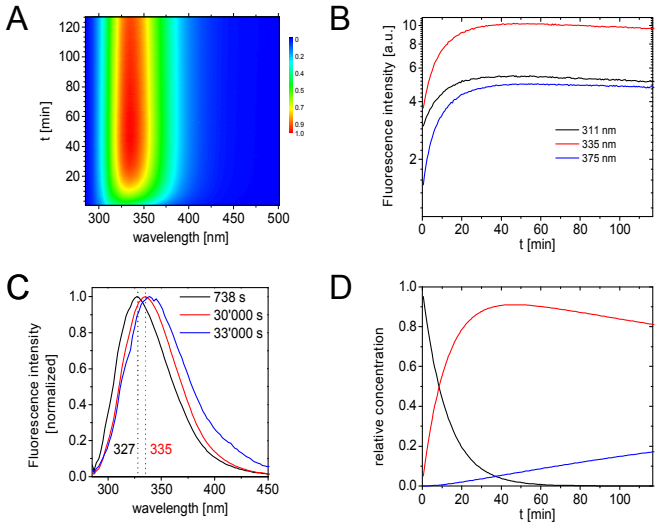

Figure 3. Fluorescence emission of UVR8 WT dimer under continuous illumination (λ_{exc} = 280 nm) (A). B: change of fluorescence emission at selected wavelengths during continuous illumination (the Y-axis is logarithmic). C: Evolution associated spectra (EAS) and their associated lifetimes. The concentration profile is given in (D).

The two slower components observed in the global analysis describe the decrease in intensity and broadening of the emission.

Surprisingly, the illumination dependent intensity increase of the emission is also present in the R286A mutant, which suggests that this particular process is not directly related to the oligomeric state of UVR8. However, in contrast to WT the intensity increase is almost irreversible (Figure 4B, S2). Again as in WT, upon further illumination a decrease of fluorescence intensity is observed.

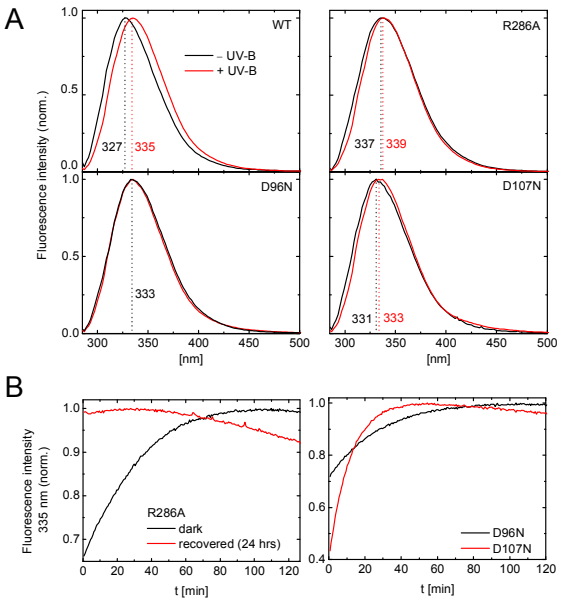

Figure 4. Tryptophan emission spectra (λ_{exc} = 280 nm) of UVR8 and selected mutants before (black) and after (red) UV-B illumination (A). Illumination dependent fluorescence intensity change of R286A (left) and D96N and D107N (right) at 335 nm (B).

The two salt-bridge mutants D96N and D107N although being functional dimers also clearly differ from WT in their emission properties. Both mutants are red shifted in their dark-adapted form relative to the WT. Moreover, the D96N mutant maintains its emission maximum at 333 nm upon illumination, while D107N shows only a minor shift from 331 to 333 nm. This finding suggests that a rearrangement of the negatively charged carboxylate side chains of D96 and D107 upon monomerisation is mainly responsible for the red shift of the emission. Both mutants show the typical illumination dependent increase and, upon long-term exposure, decrease of the emission (Figure 4B, S2). The latter effect appears less pronounced in the D96N mutant.

In total, three distinct processes are observed in the photoactivation of UVR8: First, the light-induced red shift of the emission is attributed to a reconfiguration of the salt-bridge network of predominantly R286, D96 and D107 upon monomerisation. Second, the increase of fluorescence is due to a photoinduced transformation of the tryptophan side chains or their environment in the monomer. The irreversibility of this process in the constitutive monomer R286A suggests that dimerization facilitates the reversal of this process. As a third and final process we observe a decrease and slight broadening of the fluorescence upon prolonged exposure to UV-B in all here described mutants. Both processes affecting the fluorescence may account for a light induced reorientation of the tryptophan side chains in UVR8, which result in different microenvironments affecting intensity and spectral shape of the given residue. However, further experiments are needed to obtain detailed information on these processes.

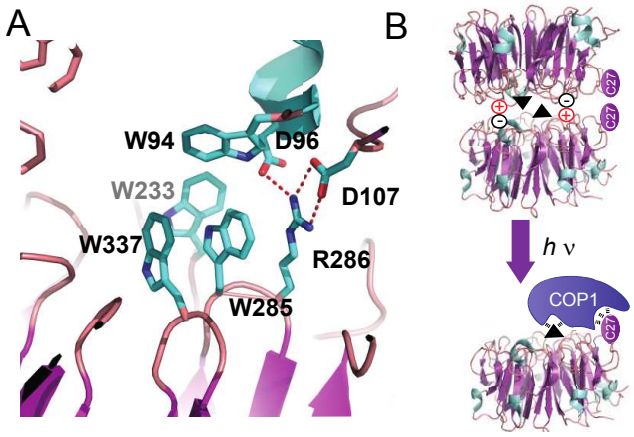

Figure 5. Molecular details of the essential salt-bridge network between R286, D96 and D107 in UVR8 close to the tryptophan pyramid (A). Photoinduced monomerisation of UVR8 enables binding to COP1 to the uncaged C27 domain and possibly another so far undefined component at the dimer interface (B).

Conclusions

Based on the spectroscopic data presented here we can infer the following model for the photoactivation mechanism of UVR8. The illumination dependent fluorescence properties of UVR8 and selected mutants show that the red shift of emission is due to a reconfiguration of the salt bridge network involving mainly R286, D96 and D107. The light induced increase of fluorescence intensity is apparently an intrinsic property of the UVR8 monomer and most likely reflects the reorganization of the environment of tryptophan side chains and could be a part of the signalling mechanism. The FTIR difference spectra of UVR8 reflect the disruption of all cross-dimer aspartate/arginine salt-bridges upon monomerisation and a concomitant minor secondary structure change. Since no apparent structural change was attributed to the deleted C-terminal region, the overall photoactivation is best described as a light-induced breaking of salt-bridges and subsequent un-caging of a so far unknown signalling element, which is required for the interaction of COP1 with the C-terminus of UVR8 (Figure 5B). Additionally, photoinduced transformations

take place in the monomer that may finally allow binding of COP1. Further studies on functional mutants and selectively isotope labelled proteins in the future will allow us to refine the tentative assignment of the structural changes presented here and to provide further insights into the molecular basis on how UVR8 can initiate signalling.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Additional FTIR spectroscopic data on H/D exchange and illumination dependent emission spectra are provided as well as limited proteolysis of UVR8.

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