Hydrogen Bond Switching among Flavin and Amino Acids Determines the Nature of Proton-Coupled Electron Transfer in BLUF Photoreceptors

Tilo Mathes^{1,2}, Jingyi Zhu¹, Ivo H.M. van Stokkum¹, M.L. Groot¹, Peter Hegemann², John T.M. Kennis¹.

¹Biophysics Group, Department of Physics and Astronomy, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1081, 1081 HV, Amsterdam, The Netherlands,

²Institut für Biologie, Experimentelle Biophysik, Humboldt Universität zu Berlin, Invalidenstraße 42,D-10115 Berlin, Germany.

Supplementary information

Experimental procedures

Sample preparation

The W91F mutation was introduced into pET28a(+)-*slr1694* as previously described and the protein was produced accordingly (Bonetti, Stierl et al. 2009). Samples for ultrafast spectroscopy were prepared and concentrated as previously described (Bonetti, Mathes et al. 2008; Bonetti, Stierl et al. 2009).

Pump-probe spectroscopy

Infrared and visible absorption spectroscopy was carried out using pump-probe setups as described earlier (Bonetti, Mathes et al. 2008; Bonetti, Stierl et al. 2009). The samples were illuminated during the measurement by a 460 nm blue LED (~ 1 W, Luxeon) to accumulate the light-adapted state and the reaction was induced at 400 nm with an energy of about 800 nJ. By checking the signal a few ps after the excitation pulse at different energies, we ensured that the excitation was not saturated. Sample concentrations were different in IR and visible experiments due to different spacer widths (200 μ m for visible, 20 μ m for IR measurements). An optical density of 0.4-0.6 at 400 nm was used. Note: excitation at 400 nm results in population of flavin S₂ and vibrationally hot S₁. This will result in a ~200 fs S₂-S₁ internal conversion and ~2 ps vibrational cooling, as described for the AppA BLUF domain (Gauden, Yeremenko et al. 2005). In the present work, these processes are not clearly observed because they are mixed with the 1 ps decay (see below).

Data analysis

Before analysis, two pre-processing methods were applied to the raw mid infrared data. The first corrects for the pre-time-zero signal introduced by thermal lens effects by calculating the average pre-time-zero signal at each wavenumber and subtracting it from the data at that wavenumber. Because no reference probe pulse was used, the noise in the measured spectra consisted mainly of so-called baseline noise (i.e., a flat, structureless offset in the spectra), which is easily recognized from a singular vector decomposition of the residual matrix, the difference between the data and the fit (van Stokkum, Larsen et al. 2004). The signal-to-noise ratio of the data was enhanced by subtracting the outer product of the first two singular vector pairs of the residual matrix (being structureless in the time domain and smooth in the wavelength domain) from the data.

The time-resolved data can be described in terms of a parametric model in which some parameters, such as those descriptive of the instrument response function (IRF), are wavenumber-dependent, whereas others, such as the lifetime of a certain spectrally-distinct component, underlay the data at all wavenumbers. The presence of parameters that underlay the data at all wavenumbers allow the application of global analysis techniques (van Stokkum, Larsen et al. 2004), which model wavenumber-invariant parameters as a function of all available data. The partitioned variable projection algorithm is well-suited to the optimization of model parameters for global analysis models (Mullen and van Stokkum 2007). The algorithm has the further advantage of estimating the standard error of parameters estimates, an advantage that is useful in model selection and validation. A compartmental model was used to describe the evolution of the spectrally distinct components in time. Global analysis was then applied to estimate the lifetime and relative concentration of each component at each wavenumber in the data.

The femtosecond transient absorption data were first globally analyzed using a kinetic model consisting of sequentially interconverting evolution-associated difference spectra (EADS), i.e. $1 \rightarrow 2 \rightarrow 3 \rightarrow \dots$ in which the arrows indicate successive mono-exponential decays of increasing time constants, which can be regarded as the lifetime of each EADS. The first EADS (SI Fig. 2) corresponds to the time-zero difference spectrum. This procedure enables us to clearly visualize the evolution of the (excited and intermediate) states of the system. In general, the EADS may well reflect mixtures of molecular states. To disentangle the contributions by the various molecular species in the spectral evolution, we performed a target analysis of timeresolved data. Target analysis involves the application of a compartmental model (i.e., a specific kinetic scheme) containing microscopic decay rates expressing intercompartmental transitions, and may be used to test detailed hypotheses regarding the underlying kinetics. The spectrum associated with each component in a target analysis applied to difference absorption data is termed species-associated difference spectra (SADS). In contrast to the EADS, the SADS will represent the spectral signature of the pure molecular species and their kinetics after photon absorption. In this way, the reaction mechanism can be assessed in terms of discrete reaction intermediate states.

The compartmental model in Fig. 3A was applied in a target analysis. The model involves 5 compartments, with the red and black compartments being spectrally identical and represent FAD*. The spectra of the starting compartments from left to right are weighted 30%, 40% and 30% in H₂O and 28%, 28% and 44% in D₂O. Only the left compartment contributes to population of compartment FADH (red), whereas the middle compartment decays entirely to the ground state. The green compartment is instantly formed and does not decay in the time scale of the experiment. It was introduced to compensate a minor offsite at the final delay time.

Results

Ultrafast spectroscopy – global analysis

Ultrafast visible absorption dynamics were recorded by pump-probe spectroscopy on Slr1694-W91F in H₂O and D₂O buffer. Both samples were measured under near-identical conditions. Slight variations after adjusting pump/probe overlap, which is necessary after replacing the sample, and slightly different sample concentrations may account for subtle differences. The obtained data was analyzed by global analysis using a sequential model $(1 \rightarrow 2 \rightarrow 3 \rightarrow ...)$. An IRF function of about 130 fs was included in the analysis as well as a 3rd order polynomial correction for the white light dispersion (a = 0.2; b = -0.1; c = -0.03). Both datasets were sufficiently described using four lifetimes. The evolution of the absorption at selected wavelengths is displayed in SI Fig. 1. At 452 nm, which is representative for the S_0 - S_1 transition of the light adapted state of Slr1694, we observe an instant bleach which decays in an isotope dependent manner. Another feature of the excited state can be observed at 556 nm, which represents the stimulated emission of the flavin. Here we observe an instant apparent bleach, which decays in parallel to the above mentioned traces. Interestingly the trace at 617 nm, which is a characteristic wavelength of a neutral flavin semiguinone, decays slower. This is even more pronounced in the corresponding D₂O traces (SI Fig. 1). In H₂O and the D₂O traces, we observe a residual absorption on each of the discussed traces, which doesn't decay on this timescale.

The corresponding evolutionary assigned difference spectra (EADS) are displayed in SI Fig 2. The first spectrum (black) corresponds to the excited state of the flavin and decays within 0.7 and 0.98 ps. The spectrum is characterized by the ground state bleach (GSB) at ~452 nm, which clearly represents the bleach of the red shifted, light adapted state. Additionally we observe excited state absorption (ESA) between 490 nm and 540 nm as well as between 590 nm and 730 nm. Additionally we see a small amount of stimulated emission (SE) at around 560 nm. The excited state evolves into the red spectrum, which lives for 4.1 / 6.6 ps. This spectrum contains the GSB at the same wavelength, but with less intensity, indicating a deactivation to the ground state. Additionally we observe a rise of the ESA between 490 nm and 540 nm. The ESA at 520 nm is slightly blue shifted. Together with the new absorptive features this indicates the formation of a new species. The red spectrum contains the same GSB with about 8 times less intensity and a shift in the putative ESA band from 520 nm to 510 nm. Additionally we observe a broad absorptive feature between 550 and 700 nm, which is similar

to the absorption a neutral flavin semiquinone. The absorption above 700 nm, however, seems uncharacteristically high. This might be due to mixing of the semiquinone spectrum with the absorption of a non-decaying species. The final, non-decaying spectrum (blue) is rather featureless with slightly rising absorbance towards the red edge of the spectral window and a bleach of the ground state absorption of the flavin.

Ultrafast IR spectroscopy – global analysis

For further characterization of the observed intermediates we performed femtosecond infrared spectroscopy. To prevent the large underlying absorption of the OH bending mode of water we measured the spectral changes between 1460 and 1740 cm^{-1} in D₂O buffer. Additional data between 1330 and 1140 cm⁻¹ was recorded in H₂O buffer but was insufficient in S/N ratio for analysis. IR measurements are in general noisier than visible data because of more fluctuations of IR probe with respect to visible, more noise in the detector and lower extinction coefficient of IR bands. The data was analysed using a sequential model, identical to the analysis of the visible absorption data. An IRF of about 160 fs was included. A correction for the probe light dispersion was not necessary in this wavelength region. Selected traces are displayed in SI Fig. 3. The most prominent signals are observed at 1691 cm⁻¹ and 1549 cm⁻¹, which represent the carbonyl stretch and the C=N in and out-of-phase vibration of the flavin. Both signals decay completely on the observed timescale. The residual, nondecaying absorption is structureless and can't be interpreted. Additionally we observe the induced absorption of the carbonyl stretch at 1674 cm⁻¹, which decays simultaneously. At 1645 cm⁻¹ we observe a characteristic bleach of the aromatic ring vibration of the isoalloxazine ring, which decays in parallel. At 1628 cm⁻¹ we observe an initial rise of the absorption, which is due to the induced absorption of the ring vibration. The signal decays quickly within 5 ps and becomes negative, indicating the formation of a new species. The negative absorption then decays within the observed timescale.

The corresponding EADS (SI Fig. 2) of the IR measurements show prominent bleaches of the flavin cofactor at around 1691 and 1549 cm⁻¹ as previously observed (Bonetti, Mathes et al. 2008). The bleach at 1550 cm⁻¹ corresponding to the C=N stretch of the isoalloxazine moiety is identical to the corresponding bleach in the dark state spectra. The carbonyl stretch at around 1700 cm⁻¹ is downshifted, as expected, by about 10 wavenumbers to 1691 cm⁻¹ in respect to the dark state spectrum. This weakening of the carbonyl bonds, indicating a stronger hydrogen bonding at the flavin carbonyl groups, is in line with observations on the light adapted state made by FTIR before. Already in the raw data we observe the formation of

a new species as indicated by a bleach that occurs within a few picoseconds after excitation. This bleach situated at around 1628 cm⁻¹ was previously assigned to the tyrosine ring vibration. This bleach was interpreted as a loss of an electron and a proton and is in line with the formation of a neutral semiquinone as observed by visible spectroscopy. The SADS (Fig. 2C) were obtained by simultaneous analysis of the visible data in D₂O and the IR dataset. Also here, we observe two identical excited state spectra and a spectrum of the radical intermediate. The SADS of the radical intermediate is very similar to the 172 ps EADS of the sequential analysis except for a small shift of the aromatic ring vibration bleach at 1647 cm⁻¹ and a slight broadening of the induced absorption of the carbonyl stretch at around 1660 cm⁻¹.

Supplementary figures



Supplementary figure 1: vis absorbance change traces obtained after 400 nm excitation of light-adapted Slr1694-W91F recorded in H₂O (black) and D₂O (red). The solid trace represents the fit. The time axis is linear up to 1 ps and logarithmic after, indicated by the skewed lines. (A) represents the decay of the ground state bleach (GSB), which is slowed down in D₂O at delays greater than 2 ps. The traces at 556 nm (B) represent stimulated emission (SE) of the flavin, which is compensated within the first 2 ps by semiquinone absorption. The absorbance change is similarly slowed down in D₂O at delays great than 2 ps. Characteristic absorption of the flavin neutral semiquinone is observed at 617 nm (C), which is biased by overlap with SE within the first 10 ps. This leads to an apparent slowed down formation of the semiquinone in D₂O, which is due to the longer lived non-productive excited state in D₂O.



Supplementary figure 2: EADS of the vis dataset in H_2O (A), D_2O (B) and the IR dataset (C). Four components were used to sufficiently fit the data in the visible datasets. The first EADS in A and B shows a clear signature of a flavin excited state featuring the GSB at 450 nm, SE at around 550 nm and ESA at above 620 nm. In both H_2O and D_2O the spectra evolve into the red EADS that does not provide any SE feature anymore, but shows partly the characteristic absorption of a flavin radical. The red spectra evolve into the green, which almost perfectly resembles the spectrum of a neutral flavin semiquinone, which decays into a minor nondecaying spectrum. This spectrum might be attributed to a solvated electron species or a minor amount of flavin triplet but contributes very little to the dataset. The IR dataset (C) was sufficiently described using three components. The first EADS shows clear features of a flavin excited state and evolves into the red EADS with a loss of absorption throughout the spectrum. Strikingly, a new bleach appears at around 1628 cm⁻¹, which is indicative of an aromatic ring vibration signature. The final non-decaying spectrum is rather featureless and cannot be interpreted.



Supplementary figure 3: IR absorption traces at selected wavenumbers. The fit is displayed as a dashed curve. The time axis is linear until a delay of 1 ps and logarithmic after. The traces at 1691 cm⁻¹ and 1674 cm⁻¹ represent bleach and induced absorption of the carbonyl groups of the flavin, whereas 1645 cm⁻¹ and 1628 cm⁻¹ can be attributed to aromatic ring (ring I) signals of the flavin, accordingly. The trace at 1549 cm⁻¹ represents the bleach of the C4a-N5/C10a-N1 out of phase vibration. All traces contain contributions of the flavin excited state and decay in parallel. The trace at 1628 cm⁻¹, however, shows a faster decay and even turns from an induced absorption signal into a bleach, thus representing a species other than the flavin.

References

- Bonetti, C., T. Mathes, et al. (2008). "Hydrogen Bond Switching among Flavin and Amino Acid Side Chains in the BLUF Photoreceptor Observed by Ultrafast Infrared Spectroscopy." <u>Biophysical Journal</u> **95**(10): 4790-4802.
- Bonetti, C., M. Stierl, et al. (2009). "The Role of Key Amino Acids in the Photoactivation Pathway of theSynechocystisSlr1694 BLUF Domain." <u>Biochemistry</u> **48**(48): 11458-11469.
- Gauden, M., S. Yeremenko, et al. (2005). "Photocycle of the flavin-binding photoreceptor AppA, a bacterial transcriptional antirepressor of photosynthesis genes." <u>Biochemistry</u> **44**(10): 3653-3662.

- Mullen, K. M. and I. H. M. van Stokkum (2007). "TIMP: An R package for modeling multiway spectroscopic measurements." Journal of Statistical Software 18(3).
- van Stokkum, I. H. M., D. S. Larsen, et al. (2004). "Global and target analysis of timeresolved spectra." <u>BBA-Bioenergetics</u> 1657(2-3): 82-104.