Photodynamics of Photo-Activated BLUF Coupled Endonuclease III Mutant RmPAE from Mesophilic, Pigmented Bacterium Rubellimicrobium mesophilum Strain MSL-20T

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Abstract
The pink to light reddish-pigmented bacterium Rubellimicrobium mesophilum strain MSL-20T contains a BLUF coupled endonuclease III of unknown function. A purified recombinant triple mutated sample of the BLUF coupled endonuclease III (F5Y, N27H, A87W) named RmPAE (Rubellimicrobium mesophilum Photo-Activated Endonuclease) was produced and characterized by optical spectroscopic methods. The BLUF domain photo-cycling dynamics occurred with high efficient blue-light induced signaling state formation (quantum yield of signaling state formation  $\phi_s = 0.6$), small spectral red-shift ($\Delta \lambda_s = 5.4$ nm), and slow thermal activated dark recovery to the receptor state ($\tau_m \approx 20$ min at room temperature). An apparent RmPAE melting temperature of $\theta_m = 63$ °C was determined by stepwise sample heating and absorption spectrum analysis. The photo-degradation of RmPAE in the signaling state was determined by prolonged intense blue-light exposure. An irreversible flavin photo-degradation occurred with quantum yield of $\phi_s = 2.6\times10^{-5}$. Schemes of the photo-cycling and the photo-degradation dynamics are presented. Engineered RmPAE may find application as light guided DNA cutter in optogenetic applications.

Introduction
The pink to light reddish-pigmented alpha proteobacterium Rubellimicrobium mesophilum strain MSL-20T (DMS 19309T, KCTC 22012T) was isolated from a soil sample of Bigeum island in Korea [1]. R. mesophilum strain MSL-20T cells are Gram-negative, motile, non-flagellated, irregular rods (size 0.4-0.7 × 1.6-3.4 µm) as characterized in [1]. R. mesophilum belongs to the abundant marine Roseobacter lineage [2]. The genome sequence of R. mesophilum strain MSL-20T is reported in [3]. It consists of 4,927,676 base pairs. The total number of genes is 5138, of which 2915 are protein-coding genes. One of these genes is coding for BLUF coupled restriction Endonuclease III (BLUF-EndoIII). It was identified through metagenome analysis using a BLUF domain containing protein as a template using Conserved Domain Search tool in NCBI portal [4]. The schematic BLUF and Endonuclease III structure is shown in Fig.1a, and the primary amino acid sequence of BLUF-EndoIII is shown in Fig.1b.

The BLUF-EndoIII protein was analyzed for flavin chromophore binding residues. A sequence alignment was carried out between the BLUF domain of BLUF-EndoIII and the BLUF domain of AppA from Rhodobacter sphaeroides [5] using BioEdit software [6]. The result is shown in Fig.1c. The conserved residues are highlighted by white letters in grey background and similar residues are highlightned by black letters in grey background. The vertical arrows indicate flavin binding amino acids. At the flavin binding positions 5, 27 and 87 the amino acids of AppA and BLUF-EndoIII are different. Especially at position 7 AppA has a Tyr residue while BLUF-EndoIII has a Phe residue and there is missing any Tyr residue in BLUF-EndoIII. Tyr residues adjacent to flavin are essential for the typical photo-cycling action of BLUF domains (for reviews see [7-9]) and the photo-activation of BLUF domain coupled cyclases (PAC proteins) (see [10] and references therein). Therefore here the BLUF domain of BLUF-EndoIII was triple mutated to BLUF-EndoIII F5Y, N27H, A87W. This engineered mutated protein is a putative photo-activated endonuclease (PAE protein) and is named RmPAE (photo-activated endonuclease from R. mesophilum).

In this paper the expression of RmPAE is described and the photo-physical dynamics is studied in detail. Different to other BLUF and PAC proteins RmPAE exhibits smaller signaling state spectral red-shift, slower signaling state recovery to the receptor state, and in the signaling state part of the BLUF domain active flavin (likely the FAD fraction) is released to BLUF domain inactive

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(improper bound or domain released) flavin. The receptor-state signaling-state photo-cycling dynamics is presented in a refined photo-cycle scheme and a ground-state and excited-state reaction coordinate scheme. Energetic and kinetic parameters are extracted. Preliminary functional characterization of the RmPAE gene paves way to develop a novel optogenetic tool.

**Experimental**

**Protein Sample Preparation and Biochemical Characterization**

**Bioinformatic analysis of BLUF-Endonuclease III:** The gene coding for BLUF coupled Endonuclease III from *R. mesophilum*, BLUF-EndoIII, was identified through metagenome analysis using a BLUF domain containing protein as template. Domain analysis of the putative BLUF-EndoIII was done using Conserved Domain (CD) Search tool at NCBI portal ([http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi](http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)) [4]. The protein BLUF-EndoIII was analyzed for flavin chromophore binding residues. Sequence alignment was carried out between BLUF domains of BLUF-EndoIII and AppA from *Rhodobacter sphaeroides* [5], (PDB Id: 1YRX) using BioEdit software [6].

**Generation and cloning of mutated BLUF-Endonuclease III (RmPAE):** Codon optimized gene of BLUF-EndoIII was synthesized for expression in *Escherichia coli* from BIOLINKK, New Delhi, India. Three residues were mutated in the synthesized gene (F5Y, N27H and A87W). These mutations were based on sequence analysis with AppA protein from *Rhodobacter sphaeroides*, (PDB Id: 1YRX) in order to obtain a photo-biologically active BLUF domain. The gene was cloned into pASKIBA43PLUS vector (IBA, Göttingen, Germany) between BamH1 and Xho1 restriction sites.

**Heterologous expression and purification of RmPAE in E. coli:** Hexa-histidine tagged mutated BLUF-EndoIII pASK construct was transformed into BL21 (DE3) cells and grown in terrific broth medium (TBM) at 37 °C till the optical density at 600 nm reached 0.8. The cells were induced with 0.02 mg/ml anhydrotetracycline (Cat. No. 2-0401-002, Nova Biological Systems Pvt. Ltd, Novabiosys, India) for 4 h at 16 °C in dark. The recombinant protein was purified from the soluble fraction with Co²⁺ IMAC resin (Clontech, Takara Bio Company) according to the manufacturer's instructions. The purified protein was dialyzed against pH 7.5 phosphate buffer consisting of 10 mM Na₂HPO₄/NaH₂PO₄ and 10 mM NaCl. SDS-PAGE and immunoblotting of
Fluorescence lifetime measurements were performed using second times after excitation light switch-off. Fluorescence spectroscopic measurements were carried out with a spectrophotometer (Cary 50 from Varian). Attenuation coefficient spectra were calculated by the relation \( \alpha(\lambda) = \ln|T(\lambda)|/\ell \), where \( \ell \) is the sample length. The attenuation coefficient \( \alpha \) is composed of absorption, \( \alpha_{\text{abs}} \), and scattering, \( \alpha_{\text{sca}} \), contributions according to \( \alpha(\lambda) = \alpha_{\text{abs}}(\lambda) + \alpha_{\text{sca}}(\lambda) \).

Fluorescence spectroscopic measurements were carried out with a spectrofluorimeter (Cary Eclipse from Varian). Fluorescence quantum distributions \( E_\lambda(\lambda) \) were determined from fluorescence emission spectrum measurements at fixed excitation wavelengths \( \lambda_{\text{exc}} \) [11,12]. The dye rhodamine 6G in methanol was used as reference standard for calibration (\( \phi_{\text{ref}} = 0.94 \) [13]). The fluorescence quantum yield \( \phi_F \) is determined by the relation \( \phi_F(\lambda) = \int E_\lambda(\lambda) d\lambda \) where the integration runs over the emission wavelength region.

For absorption spectroscopic photo-cycling investigations, RmPAE samples were brought to the signaling state (light-adapted state) by exposure with a light emitting diode (LED 455 nm from Thorlabs). The sample solution in a 1.5×3×5 mm³ cell was irradiated with the LED transverse to the transmission detection path (exposed area 3×5 mm², sample thickness along excitation path 1.5 mm, sample thickness along transmission detection path 3 mm). The excitation power \( P_{\text{exc}} \) was measured with a power meter (model PD 300-UV-SH photodiode detector head with NOVA power monitor from Ophir), and the excitation intensity \( I_{\text{exc}} \) was calculated \( I_{\text{exc}} = P_{\text{exc}}/A_{\text{cell}}, A_{\text{cell}} = 0.15 \text{ cm}^2 \).

The fluorescence spectrum recovery after signaling state formation was studied by transferring the RmPAE sample after photo-excitation in the Cary 50 spectrophotometer to the Cary eclipse fluorimeter and measuring fluorescence emission spectra at certain times after excitation light switch-off.

Fluorescence lifetime measurements were performed using second harmonic light pulses of a mode-locked titanium sapphire laser (Hurricane from Spectra-Physics, wavelength 400 nm) for sample excitation. The fluorescence signal was detected with either a micro-channel-plate photomultiplier (Hamamatsu type R1564U-01, used laser pulse duration = 3 ps, time-resolution = 500 ps) or an ultrafast streak camera (type C1587 temporal disperser with M952 high-speed streak unit from Hamamatsu, used laser pulse duration = 1 ps, time resolution = 10 ps).

The thermal stability of the protein was investigated by stepwise sample heating up and then cooling down whereby transmission spectra were measured. The apparent protein melting temperature of RmPAE was determined by analysis of the occurring absorption spectral changes [14]. Heating up to 85.2 °C caused complete irreversible protein denaturation with flavin cofactor release. The flavin cofactor composition (FAD, FMN and/or riboflavin) was estimated from fluorescence quantum yield measurements after the sample heating-cooling cycle [10].

The photo-degradation of RmPAE in the signaling state was studied by sample exposure with our light emitting diode (LED 455 nm from Thorlabs) as in the case of the photo-cycling studies but with higher excitation intensity and longer exposure time.

## Results

### Biochemical Characterization

The BLUF coupled Endonuclease III from *R. mesophilum* was analyzed for studying domain organization of the protein. Upon conserved domain analysis, light sensitive BLUF domain was found at the N-terminus spanning from 2-89 amino acids, which was coupled to the C-terminus endonuclease domain located between 113-172 amino acids (see Fig.1a). The Endonuclease III from *R. mesophilum* was found to belong to the Endonuclease III super-family which includes endonuclease III (DNA-(apurinic or apyrimidinic site) lyase), alkylase DNA glycosylases (Alkafamily) and other DNA glycosylases.

Multiple sequence alignment was performed between the BLUF domains from BLUF-EndoIII and AppA protein from *Rhodobacter sphaeroides*. This was done to analyze the conservation of key amino acids involved in flavin binding with respect to a canonical BLUF protein in order to ascertain a potentially active photoreceptor. The residues that line the flavin binding pocket in the BLUF-EndoIII are F5, S7, N27, N28, P29, L37, F44, Q46, I62, R67, H68 and A87, (see Fig.1c). On comparison with AppA protein from *R. sphaeroides*, three residues of BLUF-EndoIII namely, F5, N27 and A87 were found to be different to their corresponding residues in AppA protein. The codon optimized gene synthesized for BLUF-EndoIII had F5Y, N27H and A87W mutations (this triple mutated BLUF-EndoIII is called RmPAE).

The triple mutated BLUF-EndoIII gene was cloned between BamH1 and Xho1 into the pASK vector (see top part of Fig.2a). The 6x His-tagged BLUF-EndoIII was heterologously expressed in *E. coli* upon anhydrotetracycline induction and the purified soluble protein was detected as a ~25 kDa band upon immunoblotting, (see bottom...
part of Fig.2a). The protein was dialyzed against pH 7.5 phosphate buffer consisting of 10 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ and 10 mM NaCl.

The enzymatic activity of the triple mutated BLUF-EndoIII was examined. Since BLUF-EndoIII is an endonuclease belonging to a superfamily that includes DNA lyase, alkylation DNA glycosidases and other DNA glycosidases, we checked for the enzyme activity by looking for DNA cleavage products visible on gel. The purified photoreceptor coupled enzyme was added to DNA construct with and without appropriate buffer (e.g. FastDigest™ buffer) in the presence of blue light and the reaction products were analyzed on DNA gel. pASK DNA construct was digested by the enzyme during incubation time (first and second row of Fig.2b) as is seen by comparison with the gel trace of the pASK DNA construct alone (third row of Fig.2b). Triple mutated BLUF-EndoIII was found to be digesting/degrading the pASK vector.

Future perspectives of this novel BLUF coupled endonuclease include the determination of the mechanism of its light-gated activity.

**Spectroscopic characterization of unexposed RmPAE**

**Absorption behavior**: The attenuation coefficient spectrum $\alpha(\lambda)$ of a fresh thawed dark-adapted RmPAE sample is displayed by the solid curve in the top part of Fig.3. It was obtained by sample centrifugation with 4400 rpm for 15 min in an Eppendorf centrifuge 5702R at 4 °C. The dotted curve in the top part of Fig.3 shows the approximate scattering contribution $\alpha_{sca}(\lambda)$. It was determined by an empirical power law fit [15] of $\alpha_{sca}(\lambda) = \alpha_{sca}(\lambda_0) (\lambda_0/\lambda)^\gamma$ with $\lambda_0 = 800$ nm, $\alpha_{sca}(\lambda_0) = 0.0065$ cm$^{-1}$, and $\gamma = 4$ ($\gamma \leq 4$ depends on...
the scattering particle size with smaller γ for larger particle size). The dashed curve shows the approximate absorption coefficient spectrum contribution $\alpha_{\text{abs}}(\lambda) = \alpha(\lambda) - \alpha_{\text{sc}}(\lambda)$. For comparison the dash-dotted curve shows the absorption coefficient spectrum of FMN in aqueous solution at pH 7 normalized to the same absorption coefficient as the RmPAE sample at $\lambda = 460$ nm. For $\lambda > 310$ nm the absorption spectrum of RmPAE is determined by the flavin cofactor absorption. Below 310 nm the RmPAE apoprotein absorption contributes to the absorption mainly due to Tyr, Trp and Phe absorption (1 RmPAE protein molecule contains 1 Tyr, 1 Trp and 4 Phe residues). The absorption cross-section spectra $\sigma_{\text{abs}}(\lambda)$ of FMN in aqueous solution at pH 7 (solid curve, from [16]), FAD in aqueous solution at pH 7 (dashed curve, from [17]), Trp (dotted curve, from [18]), Tyr (dash-dotted curve, from [18]), and Phe (dashed triple-dotted curve, from [18]) are displayed in the bottom part of Fig.3.

The flavin $S_{-1}$ absorption band of RmPAE exhibits vibronic structure indicating an ordered arrangement of flavin in the BLUF domain. The long-wavelength absorption tail of flavin in RmPAE is steep. On the contrary the absorption spectrum of FMN in neutral aqueous solution is smeared out with a less steeply rising long-wavelength absorption tail due to inhomogeneous broadening in the unstructured water solution.

The flavin number density $N_{\text{Fl},0}$ of the centrifuged sample was determined by equating the $S_{-1}$ absorption cross-section integral of the flavin in RmPAE, $\int_{S_{-1}} \sigma_{\text{abs},\text{FMN}}(\mathbf{v}) d\mathbf{v}$, to the $S_{-1}$ absorption cross-section integral of FMN (flavin mononucleotide) in aqueous solution at pH 7 (from [16]), $\int_{S_{-1}} \sigma_{\text{abs},\text{FMN}}(\mathbf{v}) d\mathbf{v}$, where $\mathbf{v} = \frac{\lambda}{C}$ is the wavenumber. This equal setting gives flavin number density

$$N_{\text{Fl},0} = \int_{S_{-1}} \sigma_{\text{abs},\text{FMN}}(\mathbf{v}) d\mathbf{v} / \int_{S_{-1}} \sigma_{\text{abs},\text{FMN}}(\mathbf{v}) d\mathbf{v}$$

Using $S_{-1}$, upper wavelength borders of $\lambda_{\text{Fl},\text{RmPAE}} = 396$ nm and $\lambda_{\text{Fl,FMN}} = 399$ nm we get $\int_{S_{-1}} \sigma_{\text{abs},\text{FMN}}(\mathbf{v}) d\mathbf{v} = 8192.85$ cm$^{-2}$ and $\int_{S_{-1}} \sigma_{\text{abs},\text{FMN}}(\mathbf{v}) d\mathbf{v} = 1.71 \times 10^{-13}$ cm giving $N_{\text{Fl},0} = 4.79 \times 10^{16}$ cm$^{-3}$ (concentration $C_{\text{Fl,0}} = 7.96 \times 10^{-5}$ mol dm$^{-3}$).

The flavin loading factor of RmPAE, $\kappa_{\text{load}}$, is obtained from $N_{\text{Fl},0}$ and $N_{\text{RmPAE apoprotein}}$ to be $\kappa_{\text{load}} = N_{\text{Fl},0}/N_{\text{RmPAE apoprotein}} = 0.26$. On the average roughly each fourth RmPAE apoprotein non-covalently binds a flavin cofactor molecule. The RmPAE proteins form nano-clusters as is indicated by the RmPAE scattering coefficient spectrum $\alpha_{\text{sc}}(\lambda)$ (dotted curve in top part of Fig.3). An estimate of the average cluster size is given in section S1 of the Supplementary material (average number of RmPAE proteins in a nano-cluster is calculated to be $\beta_m = 106$).

**Fluorescence behavior:** Fluorescence quantum distributions $F_\text{F}(\lambda)$ of a fresh unexposed RmPAE sample (only used above in Fig.3 for absorption spectra measurements) are shown by solid curves in Fig.4 for fluorescence excitation wavelengths $\lambda_{\text{exc}} = 450$ nm (top part), 350 nm (middle part), and 270 nm (bottom part). The corresponding fluorescence quantum yields $\phi_F(\lambda)$ are listed in the legends of the subfigures. It should be noticed that the sample exposure for fluorescence signal measurement causes some BLUF domain signaling state formation and accompanied proper non-covalently bound flavin release to improper positioned flavin of increased fluorescence efficiency within the signaling-state lifetime (see below).

For $\lambda_{\text{exc}} = 450$ nm the fluorescence results from emission of excited non-covalently bound fully oxidized neutral flavin in the BLUF domain (peak position around 495 nm) and free fully oxidized neutral flavin (peak position around 530 nm, for different redox states and ionization states of flavins see [19]). The obtained fluorescence quantum yield is $\phi_F(450$ nm) = 0.0065±0.0005. The fluorescence quantum yield of non-covalently bound flavin cofactor is strongly quenched by BLUF-type reductive electron transfer from Tyr to photo-excited flavin (see [10] and references therein).

For $\lambda_{\text{exc}} = 350$ nm again non-covalently bound flavin and free flavin emission is observed for $\lambda > 480$ nm. Additionally a short wavelength tail extending down to 370 nm is present. It may be

![Image](https://example.com/figure3.png)

**Figure 3:** Top part: Attenuation coefficient behavior of a sample of RmPAE in pH 7.5 phosphate buffer. Bottom part: absorption cross-section spectra of FMN in aqueous solution at pH 7, FAD in aqueous solution at pH 7, Trp, Tyr, and Phe.

due to nano-cluster color-center emission [20,21], to lumichrome contribution [22], and to contribution of quinoxaline hydrolysis products of flavin [23] (likely QO1 and QO4 of [23]). The combined fluorescence quantum yield is $\phi_r(350 \text{ nm}) = 0.00879 \pm 0.0005$.

For $\lambda_{\text{exc}} = 270 \text{ nm}$ Tyr (shoulder at 308 nm), Trp (peak at 335 nm) and flavin (peak around 524 nm) contribute to the fluorescence emission. The combined fluorescence quantum yield is $\phi_r(270 \text{ nm}) = 0.0192 \pm 0.001$. The Tyr emission is strongly quenched (Tyr shoulder small compared to Trp peak) due to efficient Förster-type [11] Tyr to Trp energy transfer (see supporting information to [24]). The Trp emission is thought to be quenched by efficient Förster-type energy transfer to flavin (fluorescence quantum yield of Trp in aqueous solution is $\phi_r = 0.13$ [25]).

The dependence of the fluorescence quantum distribution of the investigated unexposed RmPAE sample on the fluorescence excitation wavelength is presented in detail in Fig.5a of the Supplementary material. There spectra are shown for fluorescence excitation wavelengths in the range from 470 nm to 230 nm in steps of 10 nm. The dependence of the corresponding fluorescence quantum yield on the fluorescence excitation wavelength is shown in Fig.S2 by the solid line connected circles. For $\lambda_{\text{exc}} \geq 470 \text{ nm}$ the fluorescence efficiency increased because of dominant absorption of free flavin. The presence of lumichrome, quinoxaline hydrolysis products (QO1, QO2, QO3, QO4 of [23]) and possible nano-cluster color center increases the fluorescence efficiency between 410 nm and 320 nm. For $\lambda_{\text{exc}} \leq 310 \text{ nm}$ the fluorescence is dominated by Trp emission. The highest fluorescence quantum yield is observed for $\lambda_{\text{exc}} = 300 \text{ nm}$ and 290 nm where the absorption is dominated by Trp which has the strongest fluorescence efficiency.

A temporal fluorescence trace (average of 10 measured traces) of a dark-adapted centrifuged RmPAE sample measured at $\theta = 21.3 \degree \text{C}$ with our microchannel-plate photomultiplier tube is displayed in the top part of Fig.5a (thick solid curve, maximum signal height is normalized to 1, i.e, $S_{\text{ftd}}(t)/S_{\text{ftd,max}}$ is presented) together with the system response function (dotted line, Rayleigh scattered light at excitation wavelength from a cell filled with water was registered). This sample was previously only exposed for an absorption spectrum measurement (negligible excitation intensity [24]) and three fluorescence spectra measurements ($\lambda_{\text{exc}} = 450 \text{ nm}$, 350 nm and 270 nm, non-negligible excitation intensity for partial signaling state formation [24]). The fluorescence trace exhibits the presence of a free flavin contribution shown by the dashed curve (convoluted single-exponential trace of amplitude fraction $d_{\text{free}} = 0.124$ and fluorescence lifetime $\tau_{\text{free}} = 5 \text{ ns}$, see section S3 of Supplementary material for the applied convolution analysis). The solid curve in the bottom part of Fig.5a shows the fluorescence contribution of the non-covalently bound flavin $S_{\text{ftd,ftb}}(t)/S_{\text{ftd,max}}$. This fluorescence trace fits well to a convoluted bi-exponential fluorescence decay (dash-dotted curve) according to

$$S_{\text{F,b,ftb}}(t) = x_{\text{b,ftb}} \exp(-t/\tau_{\text{F,b,ftb}}) + x_{\text{b,ftb}} \exp(-t/\tau_{\text{F,b,ftb}})$$

(see Eq.S5) with $x_{\text{b,ftb}} = 0.901 \pm 0.02$, $\tau_{\text{F,b,ftb}} = 65 \pm 10 \text{ ps}$, $x_{\text{b,ftb}} = 0.099 \pm 0.02$, and $\tau_{\text{F,b,ftb}} = 500 \pm 100 \text{ ps}$. $x_{\text{b,ftb}}$ is the fraction of proper non-covalently bound flavin with average fluorescence lifetime $\tau_{\text{F,b,ftb}}$. $x_{\text{b,ftb}}$ is the fraction of improper non-covalently bound flavin with fluorescence lifetime $\tau_{\text{F,b,ftb}}$. The improper bound flavin is due to release of proper bound flavin in the BLUF domain signaling state (see below). The procedure of fluorescence component amplitude and fluorescence lifetime extraction by convolution fitting is explained in section S3 of the Supplementary material.

In Fig.5b a fluorescence trace of the same RmPAE sample is shown which was measured with our ultrafast streak-camera system. The solid curve in the top part of Fig.5b shows the measured normalized fluorescence trace $S_{\text{ftd,ftb}}(t)/S_{\text{ftd,max}}$ (average of 10 measured traces, temperature $\theta = 21.1 \degree \text{C}$). The dotted curve shows the approximate contribution of free flavin and improper bound flavin [$S_{\text{ftd,ftb}}(t) + S_{\text{ftd,ftb}}(t)/S_{\text{ftd,max}}]$. The dashed gives the approximate contribution of the proper bound flavin $S_{\text{ftd,ftb}}(t)/S_{\text{ftd,ftb,max}}$. The bottom part of Fig.5b a bi-exponential convolution fit of the proper bound flavin signal is presented. The solid curve is $S_{\text{ftd,ftb}}(t)/S_{\text{ftd,ftb,max}}$. The dotted curve is the system response function (measured by attenuating the excitation laser pulse and detecting it with the streak-camera). The dash-dotted curve shows the bi-exponential convolution fit curve (Eq.S3 and Eq.S5) with $x_{\text{b,ftb}} = 0.5$ (fraction of fast decaying excited flavin), $x_{\text{d,ftd}} = 0.5$ (fraction of slow decaying excited flavin), $\tau_{\text{ftd}} = 5 \text{ ps}$ (fluorescence lifetime of fast component, determined by Tyr to excited flavin in receptor state Fl$^+$, reductive electron transfer Tyr + Fl$^+$ → Tyr$^*$ + Fl$^+$ [10,26]), and $\tau_{\text{ftd}} = 78 \text{ ps}$ (fluorescence lifetime of slow component, determined by the Fl$^+$* state lifetime [10,26], see below).

The mean fluorescence lifetime of proper bound flavin in the dark adapted state is $\tau_{\text{F,b,ftb}} = \tau_{\text{F,b,ftb}} + \tau_{\text{F,b,ftb}} + \tau_{\text{F,b,ftb}} = 41.5 \text{ ps}$. The fluorescence quantum yield of proper bound flavin in

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**Figure 4:** Fluorescence quantum distributions $E_r(\lambda)$ of RmPAE under different experimental situations of unexposed sample (solid curves), sample after three photo-cycles (dashed curves), fresh sample after heat-denaturation (dotted curves), sample after photo-degradation of Fig.10 (thick dash-dotted curve), and sample after photo-degradation and heat denaturing (dash-triple-dotted curves). Top part: fluorescence excitation wavelength $\lambda_{\text{exc}} = 450 \text{ nm}$. Middle part: $\lambda_{\text{exc}} = 350 \text{ nm}$ except indicated differently. Bottom part: $\lambda_{\text{exc}} = 270 \text{ nm}$. The fluorescence quantum yields are listed in the legends.
the dark-adapted state is $\phi_{F.b.p.da} = \frac{\tau_{F.b.p.da}}{\tau_{rad.Fl}}$

The fluorescence quantum yield of the non-covalently bound flavin in unexposed RmPAE $\phi_{F.b.da}(flavin)$ is determined by subtracting the free flavin contribution from the total measured fluorescence quantum yield of dark adapted flavin, i.e

$$\phi_{F.b.da}(flavin) = \phi_{F.b.da}(flavin) \left[ \int S_{F.b.da}(t) dt - \int S_{F.fran}(t) dt \right] \int S_{F.b.da}(t) dt = 3.52 \times 10^{-3}$$

using $\phi_{F.b.da}(flavin) = 0.0065$ (solid curve in top part of Fig.4) and

$$\int S_{F.b.da}(t) dt - \int S_{F.fran}(t) dt \int S_{F.b.da}(t) dt = 0.54$$

(integrals over solid curve and dashed curve in top part of Fig.5a). The fluorescence quantum yield contribution of the free flavin is $\phi_{F.free} = \phi_{F.b.da} - \phi_{F.b.da} = 0.00298$. The fraction of free flavin $x_{Fl.free}$ is estimated by the relation $x_{Fl.free} = \phi_{F.free}/\phi_{F.denatured}$ giving $x_{Fl.free} = 0.0196$ using $\phi_{F.denatured} = 0.152$ (see below).

**Thermal investigations:** Thermal investigations of RmPAE were carried out to get information on the thermal protein stability and to determine the flavin composition. The studies are given in the Supplementary material (section S4). The apparent RmPAE protein melting temperature $\theta_m$ was determined by stepwise sample heating and observing the vibronic structure of the first absorption band [14]. A value of $\theta_m = 63 \pm 2^\circ C$ was found. The flavin composition in RmPAE was determined by fluorescence quantum yield analysis after heat-denaturation (see dotted curves in Fig.4 and detailed presentations in Fig.S4 and Fig.S5). The mole-fraction of FMN (and possible riboflavin RF) turned out to be $x_{FMN,RF} = 0.604 \pm 0.015$ and the mole-fraction of FAD was found to be $x_{FAD} = 1 - x_{FMN,RF} = 0.396 \pm 0.015$.

**Spectroscopic characterization of light exposed RmPAE**

**Absorption photo-cycling:** The photo-cyclic absorption behavior of RmPAE was studied at room temperature. Attenuation spectra were measured before, during, and after light exposure.

In a first photo-cycling experiment a fresh centrifuged sample was exposed at 455 nm with $I_{exc} = 0.0938$ W cm$^{-2}$ for a duration of 2.8 min (exposed input energy density $w_{exc} = 15.76$ J cm$^{-2}$) at a temperature of $\theta = 23.8 \pm 0.2^\circ C$. The solid curve in the top part of Fig.6 shows the attenuation coefficient spectrum of unexposed RmPAE (dark-adapted state). The dashed curve in the top part of Fig.6 shows the attenuation coefficient spectrum of RmPAE due to sample exposure at $\lambda_{exc} = 455$ nm with input excitation intensity of $I_{exc} = 0.0938$ W cm$^{-2}$ at $t_{exc} = 12$ s. For this exposure time RmPAE is already practically completely converted to the signaling state (saturated light-adapted state). The $S_0-S_1$ flavin absorption band red shift in the signaling state is $\Delta \lambda_{s,r} = 5.4$ nm.

In the bottom part of Fig.6 the absorption difference spectrum $\delta \alpha_{exc}(\lambda) = \alpha_{exc12}(\lambda) - \alpha_{exc1}(\lambda)$ is displayed. The strongest absorption change due to signaling state formation occurred at $\lambda = \lambda_{\Delta \alpha_{max}} = 482$ nm.
The photo-cycling behavior of the same RmPAE sample in a second excitation-recovery cycle one day later is displayed in Fig. S6 of the Supplementary material (section S5.1). There the sample excitation intensity at $\lambda_{ex}$ = 455 nm was $I_{ex}$ = 0.938 mW cm$^{-2}$ and the total duration of exposure was $t_{exc}$ = 5.2 min. The RmPAE signaling state recovery was followed over 66.4 min. The top part of Fig. S6 displays the temporal signaling state formation, the middle part shows the signaling state recovery to the receptor state, and the lower part displays the absorption difference between dark recovered sample and initial sample.

In order to determine the efficiency of signaling state formation and the time constant of signaling-state recovery to the receptor state. Bottom part: Attenuation coefficient difference spectrum $\alpha(\lambda_{da})$. Dashed curve: $\alpha(\lambda_{la})$ of unexposed sample (dark-adapted sample). Solid curve: attenuation coefficient spectrum $\alpha(\lambda)$ of sample exposed at 455 nm for $t_{exc}$ = 12 s with $I_{exc}$ = 93.8 mW cm$^{-2}$ (light adapted sample, RmPAE in signaling state).

The quantum yield of signaling state formation $\phi_\text{pr}$ is determined from the rise of absorption at $\lambda$ = 482 nm at the onset of light exposure (inset of Fig.7). $\phi_\text{pr}$ is defined as the ratio of the increment of length-integrated number density $\Delta N_{\text{ph.abs}}$, i.e.

$$\phi_\text{pr} = \frac{\Delta N_{\text{ph.abs}}}{\Delta N_{\text{ex}}}.$$  

(1)

The increment of length-integrated number density $\Delta N_{\text{ph.abs}}$ is given by

$$\Delta N_{\text{ph.abs}} = \frac{N_{\text{fl,0}} \ell_{exc} \Delta \alpha_{\text{pr}}}{\Delta N_{\text{ph.abs}}}.$$  

(2)

where $N_{\text{fl,0}}$ is the initial number density of flavin, $\ell_{exc}$ is the sample length in excitation direction, $\Delta \alpha_{\text{pr}}$ is the absorption coefficient change at the probe wavelength $\lambda_{pr}$ due the photon absorption $\Delta N_{\text{ph.abs}}$, and $\Delta N_{\text{ph.abs}}$ is the maximum absorption coefficient change at the probe wavelength $\lambda_{pr}$ belonging to total conversion of RmPAE in the receptor state to RmPAE in the signaling state.

The increment of absorbed excitation photons $\Delta N_{\text{ph.abs}}$ at the onset of light exposure for duration $t_{exc}$ is

$$\Delta N_{\text{ph.abs}} = \frac{t_{exc} \ell_{exc}}{h \nu_{exc}} [1 - \exp(\frac{-\alpha_{da}(\lambda_{exc}) \ell_{exc}}{t_{exc}})].$$  

(3)

Using experimental parameters of $\alpha_{da}(\lambda_{exc}) = \alpha_{da}(455 \text{ nm}) = 1.635 \text{ cm}^{-1}$, $\ell_{exc} = 0.15 \text{ cm}$, $t_{exc} = 1.7 \text{ s}$, $\Delta \alpha_{\text{pr}} = 0.04242 \text{ cm}^{-1}$, $\Delta N_{\text{ph.abs}} = 0.534 \text{ cm}^{-1}$, $I_{exc} = 9.38 \times 10^{4} \text{ W cm}^{-2}$, and $N_{\text{fl,0}} = 3.97 \times 10^{16} \text{ cm}^{-3}$ we determine

\[\text{Fig. 7: Temporal attenuation coefficient development } \alpha_{\text{exc}}(t) \text{ of RmPAE before, during and after excitation at } \lambda_{exc} = 455 \text{ nm with } I_{exc} = 0.938 \text{ mW cm}^{-2} \text{ (see main text).}\]
\[ \Delta n_{ph(abs)} = 7.938 \times 10^{14} \text{ cm}^{-2} \text{ and } \Delta N_{r} = 4.728 \times 10^{14} \text{ cm}^{-2}. \] Insertion of these values into Eq.1 gives a quantum yield of signaling state formation of \( \phi_s = 0.596. \)

After excitation light switch-off the attenuation coefficient at \( \lambda_{ev} = 482 \text{ nm} \) recovered single exponentially from the signaling state value to the receptor state value with the time constant of \( \tau_{rec} \) (24.7 °C) = 1146.3 s = 19.1 min (dash-dotted curve in main part of Fig.7).

The photo-cycling behavior of a further RmPAE sample was investigated at \( \theta = 4.1 \pm 0.1 \text{ °C}. \) The temporal attenuation coefficient development \( \alpha(482 \text{ nm}, t) \) is shown in Fig.S7 of the Supplementary material (section S5.1). The time constant of signaling state recovery to the receptor state was found to be \( \tau_{rec} \) (4.1 °C) = 129 min.

The photo-excitation of flavin caused a protein conformational change from the receptor state to the signaling state. After light switch-off the protein recovered from the signaling state conformation back to the receptor state conformation via a potential energy activation barrier \( E_A. \) The recovery follows an Arrhenius relation according to \( [14,28] \)

\[ \tau_{rec}(\theta) = \tau_{rec,0} \exp \left( \frac{E_A}{k_B \theta} \right) = \frac{h}{k_B \theta} \exp \left( \frac{E_A}{k_B \theta} \right), \] (4)

where \( \tau_{rec,0} = h/(k_B \theta) \) is the inverse attempt frequency of barrier crossing \( [14,29] \) and \( E_A \) is the activation barrier height. \( h \) is the Planck constant and \( k_B \) is the Boltzmann constant. Solving Eq.4 for \( E_A \) gives

\[ E_A \approx k_B \theta \ln \left( \frac{\tau_{rec}(\theta)}{h} \right). \] (5)

Insertion of parameters \( \tau_{rec} \) (297.85 K) = 1146.3 s and \( \tau_{rec} \) (277.25 K) = 7740 s gives \( E_A \) (297.85 K) = 1.50 \times 10^{-19} J \((\tilde{\nu}_A = E_A/(hc_\theta) = 7551 \text{ cm}^{-1})\) and \( E_A \) (277.25 K) = 1.47 \times 10^{-19} J \((\tilde{\nu}_A = E_A/(hc_\theta) = 7383 \text{ cm}^{-1})\). Within the experimental accuracy \( E_A \) is independent of the sample temperature \( (\tilde{\nu}_A = E_A/(hc_\theta) = 7470 \pm 100 \text{ cm}^{-1}) \).

Fluorescence photo-cycling

Fluorescence emission spectroscopic behavior: The RmPAE sample used for the photo-cycle studies of Fig.6 and Fig.S6 was photo-excited a third time for fluorescence spectroscopic investigations at \( \lambda_{exc} = 455 \text{ nm} \) with \( I_{exc} = 0.938 \text{ mW cm}^{-2} \) for 5.8 min (first photo-cycle: \( I_{exc} = 0.0938 \text{ W cm}^{-2} \) for 2.8 min, see Fig.6; second photo-cycle: \( I_{exc} = 0.938 \text{ mW cm}^{-2} \) for 5.2 min, see Fig.S6).

The temporal development of the fluorescence quantum yield \( \phi_t \) and of the fluorescence quantum distribution at \( \lambda = 480 \text{ nm} \) \( E_t \) (480 nm) (there dominant emission of non-covalently bound flavin in the receptor state) is displayed in Fig.8. The top part shows the applied temporal excitation intensity. The middle part shows the fluorescence quantum yield before light exposure, at the end of light exposure (30 s after light switch-off) and its temporal evolution after light switch-off. The bottom part shows the development of \( E_t \) (480 nm) before light exposure, at the end of light exposure (30 s after light switch-off) and after light switch-off.

The fluorescence quantum yield \( \phi_t \) of flavin in RmPAE decreased during light exposure since the efficiency of fluorescence emission of non-covalently bound flavin in the signaling state of RmPAE is less than that in the receptor state. After light switch-off the flavin fluorescence efficiency increased beyond the fluorescence efficiency before light exposure with a time constant of \( \tau_{rec} \) (480 nm) = 12.91 \pm 0.3 min at \( \theta = 24.5 \text{ °C} \) which is shorter than the time constant \( \tau_{rec} \) of absorption recovery. This behavior indicates a partial bound flavin rearrangement in the BLUF domain towards higher fluorescence efficiency (proper non-covalently bound flavin in the BLUF domain rearranged to improper non-covalently bound flavin during the signaling state lifetime, see below). The dependences are analyzed in the Supporting material (section S5.2).

The fluorescence quantum distribution \( E_t \) (480 nm) (bottom part of Fig.8) is determined by the fluorescence behavior of proper non-covalently bound flavin. This fluorescence quantum distribution decreased during light exposure and recovered approximately to the initial value before light exposure single exponentially with the time constant \( \tau_{E_t(480 nm)} = 21.34 \pm 3 \text{ min at } \theta = 24.5 \text{ °C}. \)

The photo-cyclic fluorescence behavior of RmPAE will be explained below including the temporal fluorescence signal (lifetime) behavior as partial release of active non-covalently bound flavin in the BLUF domain to improperly bound flavin in the BLUF domain or to released flavin from the BLUF domain (kept in the RmPAE nano-cluster) during the stay of RmPAE in the signaling state.

**Figure 8:** Photo-cycle dependence of flavin fluorescence emission. Third photo-cycle is considered. Top part: excitation intensity profile. Middle part: fluorescence quantum yield \( \phi_t(t) \). Bottom part: fluorescence quantum distribution \( E_t \) (480 nm, t).
Details of the fluorescence spectroscopic behavior of RmPAE due to photo-excitation to the signaling state are given in the Supplementary material section S5.2.

**Fluorescence Lifetime Behavior:** The fluorescence lifetime behavior of dark-adapted RmPAE was shown above in Fig.5a and 5b. The fluorescence lifetime behavior of RmPAE in the signaling state is displayed in Fig.9.

For recording fluorescence signal traces of RmPAE in the signaling state with our streak-camera system the sample was mounted in proper position before the streak-camera and it was excited laterally with our LED 455 nm light source (sample exposure with 0.8 mW cm\(^{-2}\) for 2 min immediately before fluorescence lifetime measurement, and exposure with \(I_{\text{exc}} = 63 \mu W cm^{-2}\) during fluorescence lifetime measurement). The sample temperature was \(\theta = 21.1 \, ^{\circ}C\). The fluorescence excitation occurred with picoseconds laser pulses of 1 ps duration, and 400 nm wavelength.

The solid curve in the top part of Fig.9 shows a recorded normalized fluorescence lifetime trace \(S_{\text{F,la}}(t)/S_{\text{F,la,max}}\) (average of 10 measured single traces) of RmPAE in the signaling state. The dotted curve shows the approximate contribution of free flavin and improper bound flavin \(S_{\text{F,free}}(t)+S_{\text{F,improper}}(t)/S_{\text{F,la,max}}\). The dashed gives the approximate contribution of the proper bound flavin \(S_{\text{F,proper}}(t)/S_{\text{F,la,max}}\). In the bottom part of Fig.9 a bi-exponential convolution fit of the proper bound flavin signal in the signaling state is presented. The solid curve is \(S_{\text{F,proper}}(t)/S_{\text{F,proper,la}}\). The dotted curve is the system response function (the same as in Fig.5b). The dash-dotted curve shows a bi-exponential convolution fit (Eq.53 and Eq.55) using \(S_{\text{F,la}} = X_{\text{fla}} \exp(-t/\tau_{\text{F,fla}}) + X_{\text{sl,la}} \exp(-t/\tau_{\text{F,sl,la}})\) with \(X_{\text{fla}} = 0.8, X_{\text{sl,la}} = 1 - X_{\text{fla}} = 0.2, \tau_{\text{fla}} = 1.2 \, \text{ps}\) (electron transfer time from Tyr to Fl* [10,26]), and \(\tau_{\text{sl,la}} = 60 \, \text{ps}\) (state lifetime [10,26]).

The mean fluorescence lifetime of proper bound flavin in the signaling state is \(\bar{\tau}_{\text{F,fla}} = X_{\text{fla}} \tau_{\text{F,fla}} + X_{\text{sl,la}} \tau_{\text{F,sl,la}} = 13 \, \text{ps}\). The fluorescence quantum yield of proper bound flavin in the light-adapted state is \(\phi_{\text{F,fla}} = \bar{\tau}_{\text{F,fla}} / \tau_{\text{F,fla}} = 6.8 \times 10^{-4}\) using \(\tau_{\text{rad,Fl}} = 19 \, \text{ns}\) (value determined for riboflavin in aqueous solution [27]).

**Photo-Degradation:** The photo-degradation of RmPAE in the signaling state was studied by sample expose at \(\lambda_{\text{exc}} = 455 \, \text{nm}\) with an input intensity of \(I_{\text{exc}} = 0.108 \, \text{W cm}^{-2}\) over a time period of \(t_{\text{exp}} = 102.8 \, \text{min}\). Thereby attenuation coefficient spectra were recorded at certain exposure times, and at the end of exposure the fluorescence spectral behavior was investigated. The sample temperature was \(\theta = 23.8 \pm 0.5 \, ^{\circ}C\).

In Fig.S11 of the Supplementary material (section S6) the measured attenuation coefficient spectra are displayed. In Fig.10 the corresponding absorption coefficient spectra are shown (scattering contribution approximately subtracted). The thick dotted curve in Fig.10 shows the absorption coefficient spectrum before light exposure (RmPAE in dark-adapted state). The thin solid curve belongs to an exposed input excitation energy density of \(w_{\text{exc}} = 1.125 \, \text{J cm}^{-2}\). This curve shows the absorption coefficient...
is the number density of degraded flavins Fl$_{exc}$ in the heating process. Non-covalently bound fully reduced flavin would be released from the protein in the process of protein heat denaturizing and then re-oxidized to Fl$_{ox}$ [32] which is not observed. Likely fully reduced flavin is linked to the RmPAE protein by covalent binding to the tyrosine involved in the BLUF domain photo-cycle (Fl$_{ox}$* + TyrOH → Fl + TyrOH* → FlH + TyrO, FlH + TyrO → FlH-OTyr [10,31]). The weak long-wavelength tail absorption (λ > 520 nm) may be due to a flavin–amino acid charge transfer complex [FlH…aa*] (Fl$_{ox}$* + TyrOH + aa-H → Fl- + TyrOH* + aa-H →, [FlH…aa*] + TyrOH) [33-35].

Fluorescence quantum distributions at λ$_{exc}$ = 450 nm, 350 nm and 270 nm after the photo-degradation are included in Fig.4 (thick dash-dotted curves). The curve belonging to λ$_{exc}$ = 450 nm shows that still some oxidized flavin Fl$_{ox}$ is present. The curve belonging to λ$_{exc}$ = 350 nm indicates dominant lumichrome emission (peak around 460 nm) due to Fl$_{ox}$ photo-degradation. The increased emission around 400 nm is attributed to some flavin degradation to quinoxalines (likely QO1 and QO4 of [23]). For λ$_{exc}$ = 270 nm fluorescence contributions from Tyr, quinoxalines (QO1, QO2, QO3, QO4 of [23]), lumichrome and oxidized flavin are seen. More fluorescence quantum distributions in the fluorescence excitation wavelength region from 230 nm to 480 nm are shown in Fig.S12.

Fluorescence quantum distributions at λ$_{exc}$ = 450 nm, 340 nm and 270 nm after photo-degradation and additional heat treatment for protein denaturation are included in Fig.4 (dashed-triple-dotted curves). The curve belonging to λ$_{exc}$ = 450 nm shows increased Fl$_{ox}$ emission. The fluorescence quantum yield of fresh heat-denatured RmPAE is not reached because formed non-fluorescent adduct of RmPAE apoprotein and fully reduced flavin contributes to the absorption at λ$_{exc}$ = 450 nm. The curve belonging to λ$_{exc}$ = 340 nm indicates fluorescence contributions from oxidized flavin, lumichrome, and quinoxalines. For λ$_{exc}$ = 270 nm fluorescence contributions from Tyr, quinoxalines, lumichrome and oxidized flavin are seen. The fluorescence efficiency of Tyr and Fl$_{ox}$ is reduced because of the dominant absorption of the non-fluorescent adduct of RmPAE apoprotein and fully reduced flavin at 270 nm. More fluorescence quantum distributions in the excitation wavelength range from 230 nm to 500 nm are shown in Fig.S13.

The initial quantum yield of flavin cofactor photo-degradation $\phi_D$ in the BLUF signaling state of RmPAE is estimated by analysis of the initial absorption decrease at 470 nm (Fig.10 with inset).

$$\phi_D = \frac{\Delta N_{Fl_{ox}}}{\Delta N_{ph,abs}} \frac{\Delta \alpha_{pr}}{\sigma_{pr} \Delta \lambda_{exc}}$$

$\Delta N_{Fl_{ox}}$ is the number density of degraded flavins Fl$_{ox}$ in the signaling state due to the number density of absorbed excitation photons $\Delta N_{ph,abs}$, $\Delta \alpha_{pr}$ is the absorption coefficient decrease at $\lambda_{pr}$ (470 nm), $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$. $\Delta \lambda_{exc}$ is the absorption coefficient decrease at $\lambda_{exc} (470$ nm). $\sigma_{pr}$ is the Fl$_{ox}$ absorption cross-section at $\lambda_{pr}$.
the exposed excitation energy density at the start of light exposure, $a_{exc}$ is the absorption coefficient of $\text{Fl}_{exc}$ at $\lambda_{exc}$ (455 nm). Using the attenuation coefficient curves belonging to $w_{exp} = 1.125 ~ \text{J cm}^{-2}$ and $w_{exp} = 15.75 ~ \text{J cm}^{-2}$ ($\Delta w_{exc} = 14.625 ~ \text{J cm}^{-2}$) of Fig.10 we get the parameters $\Delta a_{ex} = 0.0607 \text{ cm}^{-1}$, $\sigma_{exp} = 3.72 \times 10^{-11} \text{ cm}^2$, $\alpha_{ex} = 1.89 \text{ cm}^{-1}$ and calculate $\phi_{s} = 2.58 \times 10^{-5}$. This low value of the quantum yield of photo-degradation of the flavin cofactor in the signaling state of the BLUF domain of RmPAE shows the high photo-stability of RmPAE.

Discussion

Results obtained by the spectroscopic characterization of RmPAE are collected in Table 1. The photo-cycling dynamics and the photo-degradation of RmPAE are discussed below.

In RmPAE the flavin loading factor was determined to be $k_{Fl,load} = 0.26$ meaning that on the average four RmPAE apo-proteins share one flavin molecule. The RmPAE protein formed nano-clusters with an average cluster size of about 106 proteins aggregated together. A small fraction of $x_{Fl,free} = 0.02$ of flavins was found to be free. The flavin cofactor was found to consist of approximately 40 % FAD and 60 % FMN (and possibly riboflavin). The flavin non-covalently bound to the BLUF domain in fresh dark-adapted RmPAE, was in proper position for BLUF photo-cycling action (signaling state formation by blue light exposure, Fl positioned adjacent to Tyr for efficient photo-induced Tyr to flavin electron transfer). Blue-light photo-excitation caused signaling state formation (quantum yield of signaling state formation $\phi_{s} \approx 0.6$), and in the signaling state there occurred partial flavin repositioning (up to about 42 %) from BLUF active position to an improper BLUF inactive position with a time constant of re-localization of $\tau_{re} = 40 \text{ min}$ at room temperature.

The fraction of flavin cofactor that took part in the position replacement from BLUF photo-cycle active site to inactive site in the signaling state was roughly equal to the mole fraction of FAD in the flavin composition ($x_{FAD} = 0.396 \pm 0.015$). It is thought that the FAD cofactor (larger size than FMN or riboflavin) suffers some force in the signaling state that causes some spatial re-orienting to negligible BLUF photo-cycling activity. The fluorescence lifetime of the improper bound flavin was determined to be $\tau_{bp} = 500 \pm 100 \text{ ps}$. It should be noticed that the fluorescence lifetime of FAD varies strongly with the arrangement of the isoalloxazine group and the adenine group of FAD [17,36,37] ($\tau_{FAD} = 7 \text{ ps}$ for stacked (closed) conformation [36] and $\tau_{FAD} = 3 \text{ ns}$ for un-stacked (stretched) conformation [17]).

A scheme of the RmPAE BLUF photo-cycling dynamics of proper bound flavin $\text{Fl}_{bp}$ is displayed in Fig.11a ([38] and references therein). The photo-excitation of $\text{Fl}_{bp}$ in the BLUF receptor state to the first excited singlet state $\text{Fl}^{\ast}_{bp}$ causes electron transfer from Tyr to $\text{Fl}^{\ast}_{bp}$ ($\text{Tyr} + \text{Fl}_{bp} \rightarrow \text{Tyr}^{+} + \text{Fl}_{bp}^{\ast}$). During the $\text{Tyr}^{+} - \text{Fl}_{bp}^{\ast}$ radical ion-pair lifetime $\tau_{ET,s} = \tau_{ET} = \frac{1}{k_{ET,s,rel}}$ there occurs a BLUF domain re-conformation with hydrogen bond restructuring to BLUF, $\text{Fl}^{\ast}_{bp}$ changing to $\text{TyrOH}^{+} - \text{Fl}_{bp}^{\ast}$ with quantum efficiency $\phi_{s}$ and to $\text{Fl}_{rel}$ with quantum efficiency $1 - \phi_{s}$. The time constant of signaling state formation is $\tau_{s,f} = \frac{\tau_{ET}}{\phi_{s}}$. The anionic flavin radical $\text{Fl}^{\ast}_{bp}$ recovers to neutral $\text{Fl}_{bp}$ with time constant $\tau_{FAD} = \tau_{ET} = \tau_{CR,s}$, according to $\text{TyrOH}^{+} + \text{Fl}^{\ast}_{bp} \rightarrow \text{TyrOH} + \text{Fl}_{bp}$.

In the BLUF, signaling state part of $\text{Fl}_{bp}$ (likely FAD) is released with a time constant of $\tau_{rel}$ to $\text{Fl}_{bp}$ (likely FAD). BLUF, recovered back to BLUF, with time constant $\tau_{et}$ by thermal activated protein structure back re-conformation and hydrogen bond back restructuring (activation energy barrier $E_{a}$).

Photo-excitation of $\text{Fl}_{bp}$ in the BLUF, signaling state caused faster $\text{Tyr} + \text{Fl}_{bp}$ electron transfer (time constant $\tau_{ET,bp} = \tau_{ET}$) and charge recombination (time constant $\tau_{re,bp} = \tau_{ET,bp}$) than photo-excitation of $\text{Fl}_{bp}$ in the BLUF receptor state. The flavin radical anion $\text{Fl}^{\ast}_{rel}$ and the tyrosine radical cation $\text{TyrOH}^{+}$ combined to the reduced flavin - tyrosine adduct $\text{FlH-OTyr}$ (abbreviated Fl-Y in Fig.11a) approximately with the quantum yield of initial photo-degradation $\phi_{s}$ (for a detailed discussion see [10,31]).

The small amount of free flavin photo-degraded easily to lumichrome [16]. The absorption and emission spectroscopic studies of the RmPAE photo-degradation indicate that $\text{Fl}_{bp}$ and $\text{Fl}_{bp}$ partly degrade with low quantum yield to lumichrome and other degradation products (likely including quinoxaline hydrolysis products of flavin [23]). For convenience structural formulæ of fully oxidized flavin $\text{Fl}_{bp}$, lumichrome LC, reduced flavin – tyrosinyl adduct Fl-Tyr, and quinoxaline derivative QO1 (1,2-dihydro-2-keto-1,6,7-trimethyl-quinoxaline-3-carboxylic acid) are plotted in Fig.S14 of section S7 of the Supplementary material.
In Fig.11b an energy level and reaction coordinate scheme of the BLUF photo-cycle dynamics is depicted. The left side shows the photo-excitation of BLUF, to BLUF*, and the right side shows the photo-excitation of BLUF, to BLUF*. The excitation of BLUF, to BLUF* (\(\text{Fl}^+ + \text{Y} \xrightarrow{h\nu} \text{Fl}^* + \text{Y}^+\)) is followed by excited-state electron transfer (\(\text{Fl}^* + \text{Y} \xrightarrow{\tau_{ET}} \text{Fl}^* + \text{Y}^+\)) with electron transfer time constant \(\tau_{ET} = \tau_{ET,da}\). The energy difference \(\delta E_r\) between \(\text{Fl}^* + \text{Y}\) and \(\text{Fl}^* + \text{Y}^+\) determines the thermodynamic equilibrium between \(\text{Fl}^* + \text{Y}\) and \(\text{Fl}^* + \text{Y}^+\) and causes the bi-exponential fluorescence decay out of level \(\text{Fl}^*\) given by

\[
S_{F,r}(t) = S_{F,r,0} \left[ x_{r,da} \exp\left(\frac{t}{\tau_{F,r,da}}\right) + x_{sl,da} \exp\left(-\frac{t}{\tau_{F,sl,da}}\right)\right],
\]

with \(x_{r,da} + x_{sl,da} = 1\) and

\[
x_{sl,da} = \frac{\exp\left(-\frac{\delta E_r}{k_B \theta}\right)}{1 + \exp\left(-\frac{\delta E_r}{k_B \theta}\right)}.
\]

Solving Eq.7b for the energy level difference \(\delta E_r\) gives

\[
\delta E_r = -k_B \theta \ln\left(\frac{x_{r,da}}{1 - x_{r,da}}\right)
\]

Insertion of parameters (Fig.5b, \(x_{r,da} = 0.5\), \(\theta = 294.25\) K) gives \(\delta E_r = 0\). Similarly the energy difference \(\delta E_s\) between \(\text{Fl}^+ + \text{Y}\) and \(\text{Fl}^+ + \text{Y}^+\) is determined using Eqs.7a-7c by replacing \(\text{Fl}^*\) by \(\text{Fl}^+\). Insertion of parameters (Fig.9, \(x_{sl,da} \approx 0.2\), \(\theta = 294.25\) K) gives \(\delta E_s = 5.63 \times 10^{-21}\) J (\(\delta \tilde{V}_s = \delta \tilde{E}_s / (h \nu) \approx 253 \text{ cm}^{-1}\)).

The activation energy barrier \(E_A\) of ground-state BLUF recovery to ground-state BLUF was determined above (Eqs. 4 and 5) to be \(E_A = 1.49 \times 10^{-19}\) J (\(\tilde{V}_A = 7470 \text{ cm}^{-1}\)). Similarly, the activation barrier \(E_{A*}\) of excited-state BLUF transfer to BLUF may be determined by the time constant \(\tau_{sf}\) of photo-induced signaling state formation and the inverse attempt frequency of barrier crossing \(\tau_{sf,0} \approx h / (k_B \theta)\) [14,29]. The responsible Arrhenius relation is

\[
\tau_{sf} = \tau_{sf,0} \exp\left(\frac{E_A^*}{k_B \theta}\right) \approx h / (k_B \theta) \exp\left(\frac{E_A^*}{k_B \theta}\right)
\]

Using \(\tau_{sf} / \tau_{sf,0} = 131\) ps (\(\tau_{sf,0} = 78\) ps, \(\phi_s = 0.596\)), and \(\theta = 21.1^\circ \text{C} = 294.25\) K one gets \(\delta \tilde{V}_s = 2.72 \times 10^{-20}\) J (\(\delta \tilde{V}_s = 1367 \text{ cm}^{-1}\)).

The energy difference \(\delta E_s\) between the ground-state energy levels \(\text{Fl} + \text{Y}\) of BLUF, and \(\text{Fl} + \text{Y}\) of BLUF, determines the fractions of flavin in the signaling state \(x_s,th\) and in the receptor state \(x_r,th\) under dark-adapted conditions at temperature \(\theta\) according to

\[
x_{r,th} = \exp\left(\frac{\delta E_s}{k_B \theta}\right),
\]

Solving for \(\delta E_s\) gives

\[
\delta E_s = -k_B \theta \ln\left(\frac{x_{r,th}}{1 - x_{r,th}}\right).
\]

An upper limit of \(x_{r,th}\) is determined by the attenuation coefficient ratio of dark-adapted sample \(\alpha_{da}\) (\(\lambda_{da,max}\)) to light adapted sample \(\alpha_{la}\) (\(\lambda_{la,max}\)) at the wavelength position \(\lambda_{da,max}\) of maximum absorption change between signaling state and receptor state (see Fig.6), i.e. \(x_{r,th, upper limit} = \alpha_{da} / \alpha_{la}\). This upper limit determination assumes that the attenuation coefficient of RmPAE in the receptor state at \(\lambda_{da,max}\) is zero (i.e. \(\alpha_{BLUF} = 0\)). From the bottom part of Fig.6 we obtain \(\lambda_{da,max} = 482\) nm and from the top part of Fig.6 we obtain \(x_{r,th, upper limit} = 0.659\). Insertion of \(x_{r,th, upper limit} = 0.659\) into Eq.9b gives \(\delta E_s \geq 2.68 \times 10^{-21}\) J (\(\delta \tilde{V}_s = \delta E_s / (h \nu) \geq 135 \text{ cm}^{-1}\)).
Table 1: Parameters of RmPAE in aqueous solution at pH 7.5 (10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, 10 mM NaCl, 25% glycerol).

<table>
<thead>
<tr>
<th>Parameter</th>
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<tr>
<td>$\chi_{FMN,RF}$</td>
<td>0.604±0.015</td>
<td>Fig. S5</td>
</tr>
<tr>
<td>$\chi_{FAD}$</td>
<td>0.396±0.015</td>
<td>$\chi_{FAD} = 1 - \chi_{FMN,RF}$</td>
</tr>
<tr>
<td>$\kappa_{(\mu)}$</td>
<td>0.26±0.03</td>
<td>Fig. 3</td>
</tr>
<tr>
<td>$\theta_0$ (°C)</td>
<td>63±2</td>
<td>Fig. S3</td>
</tr>
<tr>
<td>$x_{r,free}$</td>
<td>0.0196 ±0.0002</td>
<td>Fig. 5a</td>
</tr>
<tr>
<td>$\tau_{f,free}$ (ns)</td>
<td>5.0</td>
<td>Fig. 5a</td>
</tr>
<tr>
<td>$\phi_{F,da}$</td>
<td>0.0065±0.0005</td>
<td>Fig. S2, fresh sample</td>
</tr>
<tr>
<td>$\tau_{f,da}$ (ps)</td>
<td>5±1</td>
<td>Fig. 5b, $\tau_{f,da} = \tau_{ET}$</td>
</tr>
<tr>
<td>$\tau_{s,da}$ (ps)</td>
<td>78±5</td>
<td>Fig. 5b, $\tau_{s,da} = \tau_{CR}$</td>
</tr>
<tr>
<td>$x_{da}$</td>
<td>0.5±0.1</td>
<td>Fig. 5b</td>
</tr>
<tr>
<td>$x_{f,da}$</td>
<td>0.5±0.1</td>
<td>Fig. 5b</td>
</tr>
<tr>
<td>$x_{f,pda}$ (ps)</td>
<td>41.5</td>
<td>$x_{f,pda} = x_{f,da} + x_{s,da}$</td>
</tr>
<tr>
<td>$\phi_{f,b,p,da}$</td>
<td>$= 2.2 \times 10^{-3}$</td>
<td>$\phi_{f,b,p,da} = \tau_{rad,F} / \tau_{rad,Fl}$</td>
</tr>
<tr>
<td>$\tau_{f,b,p}$ (ps)</td>
<td>500±100</td>
<td>Fig. 5a</td>
</tr>
<tr>
<td>$\tau_{f,b}$ (ps)</td>
<td>1.2±0.2</td>
<td>Fig. 9, $\tau_{f,b} = \tau_{ET}$</td>
</tr>
<tr>
<td>$\tau_{f,s}$ (ps)</td>
<td>60±5</td>
<td>Fig. 9, $\tau_{f,s} = \tau_{CR}$</td>
</tr>
<tr>
<td>$x_{f,s}$ (ps)</td>
<td>13</td>
<td>$x_{f,s} = x_{f,b,p} + x_{s}$</td>
</tr>
<tr>
<td>$\phi_{f,b,p,s}$</td>
<td>$= 6.8 \times 10^{-4}$</td>
<td>$\phi_{f,b,p,s} = \tau_{rad,F} / \tau_{rad,Fl}$</td>
</tr>
<tr>
<td>$\tau_{rad,Fl}$ (ns)</td>
<td>19</td>
<td>[27]</td>
</tr>
<tr>
<td>$\delta A$ (nm)</td>
<td>5.4±0.4</td>
<td>Fig. 6</td>
</tr>
<tr>
<td>$\tau_{s}$ (ps)</td>
<td>131</td>
<td>$x_{s} = x_{f,pda} / Z_s$</td>
</tr>
<tr>
<td>$\tau_{rel}$ (min)</td>
<td>19.1±0.5</td>
<td>Fig. 7, $\theta = 24.7$ °C</td>
</tr>
<tr>
<td>$\tau_{rec}$ (min)</td>
<td>39.8±2</td>
<td>Fig. 8, Eq. S9b, $\theta = 24.5$ °C</td>
</tr>
<tr>
<td>$I_{sat}$ (W cm$^{-2}$)</td>
<td>$1.56 \times 10^{-5}$</td>
<td>Eq. S8b, $\theta = 24.7$ °C</td>
</tr>
<tr>
<td>$\phi_0$</td>
<td>0.596±0.04</td>
<td>Eqs. 1-3</td>
</tr>
<tr>
<td>$E_a/(hc_0)$ (cm$^{-1}$)</td>
<td>7470±100</td>
<td>Eq. 5</td>
</tr>
<tr>
<td>$E_a^*/(hc_0)$ (cm$^{-1}$)</td>
<td>1367±50</td>
<td>Eq. 11b, Eq. 8</td>
</tr>
<tr>
<td>$\delta E/(hc_0)$ (cm$^{-1}$)</td>
<td>$= 0$</td>
<td>Eq. 11b, Eq. 7c</td>
</tr>
<tr>
<td>$\delta E_1/(hc_0)$ (cm$^{-1}$)</td>
<td>$= 283±20$</td>
<td>Eq. 11b, analog Eq. 7c</td>
</tr>
<tr>
<td>$\delta E_2/(hc_0)$ (cm$^{-1}$)</td>
<td>$\geq 135$</td>
<td>Eq. 11b, Eq. 9b</td>
</tr>
</tbody>
</table>
Abbreviations used in Table 1: $\chi_{\text{FMN,RF}}$ = fraction of FMN and riboflavin non-covalently bound in RmPAE. $\chi_{\text{FAD}}$ = fraction of FAD non-covalently bound in RmPAE. $\theta_m$ = apparent protein melting temperature. $x_{\text{F,free}}$ = mole fraction of free flavin. $\tau_{\text{FL,free}}$ = fluorescence lifetime of free flavin. $\phi_{\text{FL,load}}$ = complete fluorescence quantum yield of flavin in fresh dark-adapted state. $\tau_{\text{FL,da}}$ = fast fluorescence lifetime of proper bound flavin in dark-adapted BLUF domain. $\tau_{\text{FL,la}}$ = slow fluorescence lifetime of proper bound flavin in dark-adapted BLUF domain. $x_{\text{FL,da}}$ = fraction of flavins with fast fluorescence decay in dark-adapted state. $x_{\text{FL,la}}$ = fraction of flavins with slow fluorescence decay in dark-adapted state. $\phi_{\text{F,da,la}}$ = mean fluorescence lifetime of proper bound flavin in dark-adapted state. $\phi_{\text{F,la,da}}$ = fluorescence quantum yield of proper non-covalently bound flavin in the dark-adapted state. $\tau_{\text{FL,ip}}$ = fluorescence lifetime of improper non-covalently bound flavin. $\tau_{\text{FL,da}}$ = fast fluorescence lifetime of proper bound flavin in light-adapted BLUF domain. $\tau_{\text{FL,la}}$ = slow fluorescence lifetime of proper bound flavin in light-adapted BLUF domain. $x_{\text{FL,da}}$ = fraction of flavins with fast fluorescence decay in light-adapted state. $x_{\text{FL,la}}$ = fraction of flavins with slow fluorescence decay in light-adapted state. $\phi_{\text{F,da,la}}$ = mean fluorescence lifetime of proper bound flavin in light-adapted state. $\phi_{\text{F,la,da}}$ = fluorescence quantum yield of proper non-covalently bound flavin in the light-adapted state. $\tau_{\text{rad,FL}}$ = radiative lifetime of flavin (value determined for riboflavin is used [27]). $\delta\lambda_{\text{rad}}$ = red-shift of $S_0-S_1$ absorption band due to signaling state formation. $\tau_{\text{rec}}$ = signaling state recovery time of RmPAE. $\tau_{\text{rec}}$ = time constant of flavin release from proper non-covalently bound position in BLUF domain of RmPAE during presence in the signaling state. $I_{\text{rad}}$ = saturation intensity of signaling state formation for RmPAE. $\phi_{\text{s}}$ = quantum efficiency of signaling state formation. $\phi_{\text{r}}$ = quantum yield of RmPAE photo-degradation. $E_a$ = activation barrier for thermal signaling state to receptor state recovery of RmPAE. $E_a^*$ = activation barrier for signaling state formation in the excited state. $\delta E_i$ = energy difference between $\text{F}^+_i + \text{Y}^{\text{Tyr}}$ and $\text{F}^+_i + \text{Y}^{\text{Tyr}}$. $\delta E_s$ = energy difference between $\text{F}^+_i + \text{Y}^{\text{Tyr}}$ and $\text{F}^+_i + \text{Y}^{\text{Tyr}}$. $\delta E_s$ = energy difference between $\text{F}^+_i + \text{Y}^{\text{Tyr}}$ and $\text{F}^+_i + \text{Y}^{\text{Tyr}}$ in the ground-state.

Conclusions

The photodynamics of the engineered triple-mutated photovertically endonuclease RmPAE from the mesophilic, pigmented bacterium *Rubellimicrobium mesophilum* strain MSL-20T was characterized by optical spectroscopic methods. The RmPAE BLUF domain photo-cycling efficiency of receptor state to signaling state conversion was found to be reasonably high (quantum yield of signaling state formation $\phi_s = 0.6$). The first absorption band spectral red-shift of the flavin cofactor in the signaling state was rather small ($\delta\lambda_{\text{rad}} = 5.4$ nm). The RmPAE signaling-state conformation at room temperature recovered only slowly to the receptor state conformation (recovery time $\tau_{\text{rec}} = 19$ min). Because of the slow signaling state recovery, weak continuous blue light is sufficient to keep RmPAE in the saturated light-adapted state (saturation intensity at $\lambda_{\text{sat}} = 455$ nm is $I_{\text{sat}} = 16$ µW cm$^{-2}$, see section S5.1 of Supplementary material). In the signaling state part of the flavin cofactor (likely FAD) at room temperature lost its photo-cycling activity with a time constant of $\tau_{\text{rec}} = 40$ min likely by re-localization to an electron-transfer inactive site. The thermal stability of RmPAE turned out to be reasonably high (apparent protein melting temperature of $\theta_m = 63$ °C) allowing convenient stable experimentation at room temperature.

The primary BLUF domain photo-cycling behavior of RmPAE starting from the BLUF domain in its receptor state (BLUF$_s$) was similar to other BLUF proteins studied thus far [38]. The first flavin absorption band spectral red-shift in the signaling state was rather small, and the signaling state recovery to the receptor state after light switch-off was rather long. Different to other BLUF proteins, for RmPAE in the signaling state, there occurred partial removal of BLUF active flavin in proper bound position to BLUF inactive flavin in improper position (likely FAD cofactor deactivation).

Photo-excitation of RmPAE in its light-adapted signaling state (BLUF$_s$, secondary BLUF domain photo-cycling) caused permanent flavin cofactor degradation with low quantum yield of photo-degradation partly to FIH-OTyr adduct formation and partly to conversion of flavin to lumichrome and quinoxaline derivatives. A similar secondary photo-cycling dynamics with FIH-OTyr adduct formation was observed for the photovertivated adenyl cyclase LiPAC from the spirochete bacterium *Leptonema illini* strain 3055T [10] and the photovertivated adenyl cyclase TpPAC from the spirochete bacterium *Turneriella parva* strain H$^*$ [31].

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Supplementary Material to Photodynamics of photo-activated BLUF coupled Endonuclease III mutant RmPAE from mesophilic, pigmented bacterium *Rubellimicrobium mesophilum* strain MSL-20T

S1. Nano-cluster size of fresh dark-adapted RmPAE

The nano-cluster size of fresh dark-adapted RmPAE is determined analogous to the description in [20].

The scattering cross-section $\sigma_{\text{scat}}$ is obtained from the scattering coefficient $c_{\text{scat}}$ by $c_{\text{scat}} = \sigma_{\text{scat}}/N_{\text{RmPAE apoprotein}}$. For the situation of Fig.3, in the transparency region at $\lambda = 632.8$ nm it is $\alpha_{\text{scat}}(\lambda) = \alpha_{\text{scat}}(\lambda_0)(\lambda_0 / \lambda)^N = 0.0166$ cm$^{-1}$ ($\lambda_0 = 800$ nm,
\[ \alpha_{sc}(\lambda) = 0.0065 \text{ cm}^{-1}, \text{ and } \gamma = 4 \] and \( \sigma_{sc}(\lambda) = 8.97 \times 10^{-20} \text{ cm}^2 \) 
\( N_{\text{RmPAE apoprotein}} = 1.85 \times 10^{17} \text{ cm}^{-3} \).

The scattering cross-section is given by [39]
\[
\sigma_{sc} = \beta_m M \sigma_{sc,m}^{-1} 
\]
with the monomer Rayleigh scattering cross-section
\[
\sigma_{sc,m} = \frac{8 \pi n^2}{3 \lambda^4} \left( \frac{2n^2 - n_s^2}{n_s^2 + 2n_s^2} \right)^2 = \frac{8 \pi}{3} \frac{2n^2 - n_s^2}{n_s^2 + 2n_s^2} \left( \frac{M_{pr}}{N_A \rho_{pr}} \right) \left( \frac{\beta_m M}{N_{\text{RmPAE apoprotein}}} \right) \left( \frac{\sigma_{sc,m}}{\sigma_{sc}} \right) \left( N_{\text{RmPAE apoprotein}} \right) \left( \frac{\gamma}{4} \right) \left( \sigma_{sc,m} \right)
\]

Thereby \( \beta_m \) is the degree of aggregation (average number of protein molecules per cluster particle), and \( \tilde{M} \) is the total Mie scattering function (\( \tilde{M} \leq 1 \) decreasing with increasing aggregate size [39]), \( n \) is the refractive index of the solvent (water buffer) at wavelength \( \lambda \), \( n_p \) the refractive index of the protein at wavelength \( \lambda \), \( V_m = M_{pr} / (N_{\text{RmPAE apoprotein}}) \) is the volume of one protein molecule, \( M_{pr} \) is the molar mass of the protein monomer (\( M_{pr} = 24781.959 \text{ g mol}^{-1} \) for RmPAE apoprotein), \( N_a = 6.022142 \times 10^{23} \text{ mol}^{-1} \) is the Avogadro constant, and \( \rho_{pr} \) is the mass density of the protein (typical value for proteins is \( \rho_{pr} = 1.412 \text{ g cm}^{-3} \) [40]). These numbers give a protein monomer volume of \( V_m = 29.14 \text{ nm}^3 \) and a protein monomer radius of \( r_a = \left( 3V_m / (4\pi) \right)^{1/3} = 1.91 \text{ nm} \). At \( \lambda = 632.8 \text{ nm} \) there is \( n_s = 1.332 \) and \( n_p = 1.589 \) [41] giving \( \sigma_{sc,m} = 8.426 \times 10^{-22} \text{ cm}^2 \). Insertion into Eq. S1 gives \( \beta_m \tilde{M} \approx \beta_m = \sigma_{sc} / \sigma_{sc,m} \approx 106 \). The average aggregate volume is \( V_m = 3089 \text{ nm}^3 \) and the average aggregate radius is \( a_{ag} = \beta_m^{1/3} a_m = 9.04 \text{ nm} \) (for this size Rayleigh scattering applies and \( \tilde{M} = 1 \) [39]).

S2. Fluorescence excitation wavelength dependent fluorescence emission of unexposed RmPAE

In Fig. S1 fluorescence quantum distributions \( E_F(\lambda) \) of the unexposed centrifuged (4400 rpm, 15 min) RmPAE sample used in the absorption measurements of Fig. 3 are presented for fluorescence excitation in the range from \( \lambda_{F,exc} = 470 \text{ nm} \) to 230 nm in steps of 10 nm. The dependence of the overall fluorescence quantum yield \( \phi_F \) on \( \lambda_{F,exc} \) is shown by the solid curve in Fig.S2.

S3. Fluorescence amplitude contribution and fluorescence lifetime determination by fluorescence trace convolution fit

Experimental normalized fluorescence traces \( s_F(t) = S_F(t) / S_{F,\text{max}} \) are the convolution of the true \( \delta \)-function pulse excitation fluorescence decay curve \( s_{F, \delta}(t) \) and the system response function \( g(t) \) according to [27]
\[
s_F(t) = \frac{\int_{-\infty}^{t} g(t') s_{F, \delta}(t-t') dt'}{\max(\int_{-\infty}^{t} g(t') s_{F, \delta}(t''-t') dt')} \tag{S3}
\]

(the denominator with \( -\infty < t'' < + \infty \) gives the normalization to \( \max(s_F(t)) = 1 \)). For single exponential fluorescence decay \( s_{F,t}(t) \) is given by
\[
s_{F,t}(t) = \begin{cases} \exp\left(-\frac{t}{\tau_F}\right) & \text{for } t \geq 0 \\ 0 & \text{for } t < 0 \end{cases} \tag{S4}
\]

where \( \tau_F \) is the fluorescence lifetime. In the determination of the fluorescence lifetime \( \tau_{\text{fit}} \) values are inserted in Eq.S4, and \( s_{F,t}(t) \) curves are calculated by use of Eq.S3. \( s_{F,t}(t) \) is compared with the experimental \( s_F(t) \). \( \tau_{\text{fit}} \) agrees with the experimental \( \tau_F \) when \( s_{F,t}(t) \) agrees with \( s_F(t) \).
For two-component single-exponential fluorescence decay $s_{F,t}(t)$ is given by

$$s_{F,t}(t) = \begin{cases} x_1 \exp \left(-\frac{t}{\tau_{F,1}}\right) + (1-x_1) \exp \left(-\frac{t}{\tau_{F,2}}\right) & \text{for } t \geq 0 \\ 0 & \text{for } t < 0 \end{cases}$$

(S5)

$x_1$, $\tau_{F,1}$ and $\tau_{F,2}$ are adjusted to obtain a good fit of Eq.S3 to the experimentally measured fluorescence trace.

S4. Thermal investigations of fresh dark adapted RmPAE

The thermal protein stability was studied by stepwise sample heating up to 85.2 °C, then cooling down, thereby measuring the attenuation coefficient spectra development, and fluorescence characterization after the heating − cooling cycle. The apparent RmPAE protein melting temperature is derived from the loss of the vibronic structure of the $S_0$-$S_1$ absorption band of the flavin cofactor in RmPAE with sample heating [15]. The flavin composition (content of flavin-mononucleotide FMN, possible riboflavin, and flavin-adenine-dinucleotide FAD [42]) is estimated from fluorescence quantum yields measured after protein denaturing.

The heating-cooling temperature profile applied to the RmPAE sample used in the thermal investigations is displayed by the right inset of Fig.S3.

The main part of Fig.S3 shows attenuation coefficient spectra of RmPAE measured at selected temperatures during the sample heating up (19.5 °C start, 54.8 °C, 60.1 °C, 65.4 °C, 70.6 °C, 74.8 °C, 85.2 °C), cooling down (25 °C cooling down, dashed curve) and at the end of the sample cooling down and centrifugation (4 °C, end, dotted curve). The temperatures belonging to the curves are written to the curves. With rising temperature the light scattering increased (see increasing attenuation coefficient spectra) and the spectral structure of the $S_0$-$S_1$ absorption band of flavin (wavelength region from 400 nm to 500 nm) smoothed out. In cooling down the attenuation spectrum continued to increase. Only due to centrifugation the scattering reduced (dotted curve belonging to end of heating-cooling cycle with centrifugation at 4 °C). The final

$$\rho_{a,\text{mean}} = \frac{\rho_{a}(19.5^\circ C) + \rho_{a}(85.2^\circ C)}{2}$$

is determined.

The left inset of Fig.S3 shows the loss of the dip in the attenuation spectrum at $\lambda = 455$ nm with rising temperature due to protein denaturing (protein unfolding). The ratio

$$\rho_{a}(\vartheta) = 2\alpha(455\text{ nm}, \vartheta)\left[\alpha(440\text{ nm}, \vartheta) + \alpha(462\text{ nm}, \vartheta)\right]$$

is displayed (line-connected circles). The dashed line is given by

$$\rho_{a,\text{mean}} = \frac{\rho(19.5^\circ C) + \rho(85.2^\circ C)}{2}.$$ 

The apparent RmPAE melting temperature $\vartheta_m$ is defined by $\rho_{a}(\vartheta_m) = \rho_{a,\text{mean}}$. A value of $\vartheta_m = 63\pm 2^\circ C$ is determined.

The fluorescence emission quantum distributions of RmPAE after the heating-cooling cycle are shown in Fig.S4 for fluorescence excitation wavelengths in the range from 230 nm to 480 nm.

The flavin absorption coefficient at the end of the heating-cooling cycle is higher than at the start of the heating-cooling cycle since some buffer solvent evaporated at the applied high temperatures (final sample volume less than initial sample volume).

The left inset of Fig.S3 shows the loss of the dip in the attenuation spectrum at $\lambda = 455$ nm with rising temperature due to protein denaturing (protein unfolding). The ratio

$$\rho_{a}(\vartheta) = 2\alpha(455\text{ nm}, \vartheta)\left[\alpha(440\text{ nm}, \vartheta) + \alpha(462\text{ nm}, \vartheta)\right]$$

is displayed (line-connected circles). The dashed line is given by

$$\rho_{a,\text{mean}} = \frac{\rho(19.5^\circ C) + \rho(85.2^\circ C)}{2}.$$ 

The apparent RmPAE melting temperature $\vartheta_m$ is defined by $\rho_{a}(\vartheta_m) = \rho_{a,\text{mean}}$. A value of $\vartheta_m = 63\pm 2^\circ C$ is determined.

The fluorescence emission quantum distributions of RmPAE after the heating-cooling cycle are shown in Fig.S4 for fluorescence excitation wavelengths in the range from 230 nm to 230 nm in steps of 10 nm. The flavin fluorescence peaking around 535 nm is strongly increased compared to dark-adapted unheated RmPAE.
(Fig.S1) due to flavin release from the protein. The Trp fluorescence peaking around 340 nm is decreased compared to the dark-adapted unheated RmPAE (Fig.S1) probably due to the protein unfolding and aggregation. The short-wavelength fluorescence tail around 450 nm in the case of fluorescence excitation in the range from 410 nm to 320 nm may be due to lumichrome emission. The weak fluorescence emission around 400 nm in the case of fluorescence excitation between 330 nm and 310 nm may be due to emission of a quinoxaline hydrolysis product of flavin [24].

The dependence of the fluorescence quantum yield \( \phi_F \) of RmPAE after the heating-cooling cycle on the fluorescence excitation wavelength is shown in Fig.S5. In the range from 480 nm to 350 nm the fluorescence quantum yield is approximately constant at \( \phi_F = 0.152 \pm 0.003 \). In the range from 340 nm to 300 nm the fluorescence quantum yield decreases due to additional higher excited state deactivation paths (violation of Kasha-Vavilov rule of excitation wavelength independent fluorescence emission \([43,44]\)). In the range from 290 nm to 270 nm the fluorescence quantum yield is determined by flavin and Trp emission. At shorter excitation wavelength the fluorescence quantum yield is thought to be reduced by higher excited state deactivation paths for Tyr, Trp, and flavin.

The obtained fluorescence quantum yield of flavin in heat-denatured RmPAE at 450 nm is \( \phi_F = 0.152 \pm 0.003 \). Before sample heating the fluorescence quantum yield at 450 nm was \( \phi_F = 0.0065 \pm 0.0005 \). The rise in fluorescence quantum yield indicates the release of flavin in the heating process (protein denaturation).

The flavin fluorescence quantum yield \( \phi_F,\text{Fl,denatured} = 0.152 \pm 0.003 \) of heat-denatured RmPAE at the flavin fluorescence excitation wavelength of \( \lambda_{\text{exc}} = 450 \) nm is used to estimate the flavin composition in RmPAE. \( \phi_F,\text{Fl,denatured} \) is determined by the mole-fractions \( x_{\text{FMN}} \) of FMN (\( \phi_{\text{FMN}} = 0.23 \) [16]), \( x_{\text{RF}} \) of riboflavin (\( \phi_{\text{riboflavin}} = 0.26 \) [27]) and \( x_{\text{FAD}} \) of FAD (\( \phi_{\text{FAD}} = 0.033 \) [17]). It is

\[
\phi_{F,\text{Fl,denatured}} = x_{\text{FMN}} \phi_{\text{FMN}} + x_{\text{RF}} \phi_{\text{RF}} + x_{\text{FAD}} \phi_{\text{FAD}}
\approx x_{\text{FMN}} \phi_{\text{FMN}} + x_{\text{FAD}} \phi_{\text{FAD}}
= x_{\text{FMN}} \phi_{\text{FMN}} + (1 - x_{\text{FMN}}) \phi_{\text{FAD}}
\]

\((S6)\)
where \( x_{FMN,RF} = x_{RF} + x_{RF,RF} \) is the mole-fraction of FMN and RF together. From the experimental result of \( \phi_{F,RF,denatured} \), only mole-fractions \( x_{FMN,RF} \) and \( x_{RF,RF} \) can be estimated since the fluorescence quantum yield of FMN and riboflavin are nearly the same.

Rearrangement of Eq. S6 gives

\[
X_{FMN,RF} \approx \frac{\phi_{F,RF} - \phi_{F,FAD}}{\phi_{F,FMN} - \phi_{F,FAD}}
\]  

(S7)

Insertion of values gives \( x_{FMN,RF} = 0.604 \pm 0.015 \) and \( x_{RF} = 0.396 \pm 0.015 \).

### S5. BLUF domain signaling state formation and recovery in RmPAE

#### S5.1. Absorption spectroscopic investigation

The photo-cycling behavior of RmPAE in a second excitation-recovery cycle 25.75 h after the first photo-cycle of Fig. 6 is displayed in Fig. S6. The sample was excited at \( \lambda_{exc} = 455 \text{ nm} \) with \( I_{exc} = 0.938 \text{ mW cm}^{-2} \) for a duration of 5.2 min. The RmPAE signaling state recovery was followed over 66.4 min. The top part of Fig. S6 displays the temporal signaling state formation, the middle part shows the signaling state recovery to the receptor state, and the lower part displays the attenuation coefficient difference between dark recovered sample and initial sample. The absorption spectrum of the dark-adapted sample after \( t_{rec} = 66.4 \text{ min} \) is sharper than the initial absorption spectrum before light exposure. This indicates some irreversible partial protein restructuring during being in the signaling state.

The temporal attenuation coefficient development at \( \lambda_{pr} = 482 \text{ nm} \) of RmPAE at \( \theta = 4.1 \pm 0.1 \text{ °C} \) after light exposure at 455 nm with \( I_{exc} = 0.938 \text{ mW cm}^{-2} \) for \( t_{exc} = 2 \text{ min} \) is shown in Fig. S7. At \( t = -29 \text{ min} \) the sample was taken from its dark storage place at 4.1 °C and its absorption and fluorescence spectrum was measured. Then in the time range between \( t = -2 \text{ min} \) and 0 min the sample was exposed at 455 nm with \( I_{exc} = 0.938 \text{ mW cm}^{-2} \). After that the sample was kept in the dark at 4.1 °C and at the displayed time points sample attenuation spectra were measured. The attenuation coefficient recovery from the signaling state to the receptor state fits well to a single exponential curve with time constant \( \tau_{rec}(4.1 \text{ °C}) = 129 \text{ min} \).

The saturation energy density \( w_{sat} \) of light exposure to transfer RmPAE from the receptor state to the signaling state is given by [33]

\[
w_{sat} = \frac{\hbar v_{exc}}{\sigma_{abs,exc} \phi_{s}}
\]  

(S8a)

where \( v_{exc} = c/\lambda_{exc} \) is the excitation frequency, \( c \) is the speed of light in vacuum. Insertion of parameters (\( \lambda_{exc} = 455 \text{ nm} \), \( \sigma_{abs,exc} = 4.1 \times 10^{-17} \text{ cm}^2 \), \( \phi_{s} = 0.596 \)) gives \( w_{sat} = 0.0179 \text{ J cm}^{-2} \). The corresponding saturation intensity is

\[
I_{sat} = \frac{w_{sat}}{\tau_{rec}} = \frac{\hbar v_{exc}}{\sigma_{abs,exc} \tau_{rec} \phi_{s}}
\]  

(S8b)

Its value at \( \theta = 24.7 \text{ °C} \) (\( \tau_{rec} = 19.1 \text{ min} \)) is \( I_{sat}(24.7 \text{ °C}) = 15.6 \text{ µW cm}^{-2} \) and at \( \theta = 4.1 \text{ °C} \) (\( \tau_{rec} = 129 \text{ min} \)) its value is \( I_{sat}(4.1 \text{ °C}) = 2.31 \text{ µW cm}^{-2} \).

#### S5.2. Fluorescence spectroscopic investigations

Three fluorescence quantum distributions belonging to RmPAE of a third photo-cycle run (\( \lambda_{exc} = 455 \text{ nm} \), \( I_{exc} = 0.938 \text{ mW cm}^{-2} \), \( t_{exc} = 5.8 \text{ min} \), see main text) are depicted in the top part of Fig. S8. The fluorescence was excited at \( \lambda_{exc} = 450 \text{ nm} \). The dotted curve belongs to the situation before light exposure. The solid curve was measured 30 s after excitation light switch-off. It gives the fluorescence situation of the RmPAE BLUF domain in the signaling state. The dashed curve was measured 60 min after excitation light switch-off. It gives the fluorescence situation of the RmPAE BLUF domain in the recovered dark-adapted state.

The difference between the dotted curve and the solid curve is due to fluorescence emission reduction of proper bound flavin in the signaling state compared to the receptor state. The normalized difference spectrum

\[
\left| E_{F, \text{ before exposure}}(\lambda) - E_{F, \text{ before exposure} - E_{F, \text{ before exposure}} - E_{F, \text{ before exposure} - 30 s}} \right|_{\text{max}}
\]

is shown by the solid curve in the bottom part of Fig. S8. It gives approximately the shape of the fluorescence quantum distribution of flavin in the proper bound state (assuming the same shape of proper bound flavin in the receptor state and the signaling state). The difference between the dashed curve and the dotted curve in the top part of Fig. S8 is due to proper bound flavin release to improper...
bound flavin in the BLUF domain signaling state. The dashed curve in the bottom part of Fig. S8 shows the normalized difference spectrum $\left[ E_{\text{F,rec}} - 0 \text{ min } (\lambda) - E_{\text{F, precedes}} (\lambda) \right] / (E_{\text{F,rec}} - 0 \text{ min } - E_{\text{F, precedes}})_{\text{max}}$. It gives the shape of the fluorescence quantum distribution of improper bound flavin. The dotted curve in the bottom part of Fig. S8 shows the shape of the fluorescence quantum distribution of FMN in aqueous solution at pH 7. The fluorescence spectrum of proper bound flavin is approximately 27 nm blue-shifted compared to FMN, while the shape of the fluorescence spectrum of improper bound flavin roughly agrees with the shape of FMN.

The fluorescence quantum yield recovery, shown in the middle part of Fig. S8, follows the relation

$$\phi_F(t) = \phi_F(0) + \Delta \phi_F \left[ 1 - \exp \left( -\frac{t}{\tau_{\phi,rec}} \right) \right]$$  \hspace{1cm} (S9a)

where $\tau_{\phi,rec}$ is the time constant of proper non-covalently bound flavin release in the signaling state to improper bound flavin. Rewriting Eq.(9b) gives

$$\frac{1}{\tau_{\phi,rec}} \approx \frac{1}{\tau_{\phi,rel}} + \frac{1}{\tau_{\phi,rel}}$$  \hspace{1cm} (S9b)

The dependence of the flavin fluorescence quantum yield in dark-adapted RmPAE on the number of photo-cycles $n$ is depicted in Fig. S9. The circles show the development of total dark-adapted fluorescence quantum yield $\phi_{F, d a}$. The dash-dotted fit curve is given by

$$\phi_{F, d a}(n) = \phi_{F, d a}(0) + \Delta \phi_{F, d a} \left[ 1 - \exp \left( -\frac{n}{\tau_{\phi, rec}} \right) \right]$$  \hspace{1cm} (S9c)

Insertion of the values $\tau_{\phi, rec} = 19.1$ min and $\tau_{\phi, rel} = 12.91$ min gives $\tau_{\phi, rel} = 39.8$ min (temperature $\theta = 24.5^\circ$C).

- **Fig. S9:** Development of flavin fluorescence quantum yield of dark-adapted RmPAE on the number of photo-cycles.

- **Fig. S10:** Fluorescence quantum distributions of RmPAE measured 18 h after third photo-cycle (total accumulated excitation energy density in the three photo-cycles $w_{\text{exc}, \text{accum}} = 16.37 \text{ J cm}^{-2}$). The various fluorescence excitation wavelengths are given in the legends of the subfigures.

- **Fig. S11:** Attenuation coefficient spectra of RmPAE due to light exposure at 455 nm with $I_{\text{exc}} = 0.108 \text{ W cm}^{-2}$. Accumulated exposed excitation energy densities $w_{\text{exc}}$ are listed in the legend. Temperature $\theta = 23.8 \pm 0.5^\circ$C. The inset shows absorption cross-section spectra of lumichrome in aqueous solution at pH 8 (from [22]) and of quinoxaline derivatives (from [23]).
\[ \phi_{\text{F,da}}(n) = \phi_{\text{F,da}}(0) + \Delta \phi_{\text{F,da,\infty}} \left[ 1 - \exp \left( -\frac{\tau_{\text{rel}}}{} \right) \right], \]  

with \( \Delta \phi_{\text{F,da,\infty}} = 0.0101 \) and \( \tau_{\text{rel}} / \tau_{\text{rad,Fl}} = 0.48 \) (\( \tau_{\text{rel}} = 19.1 \text{ min}, \tau_{\text{rad,Fl}} = 39.8 \text{ min} \)). \( \Delta \phi_{\text{F,da}} \) is related to the fraction \( \delta_{N_{\text{F,b,p,\infty}}}/N_{\text{Fl,0}} \) of proper non-covalently bound flavin released in an infinite number of photo-cycles (\( n \to \infty \)) by

\[ \Delta \phi_{\text{F,da,\infty}} = \frac{\delta_{N_{\text{F,b,p,\infty}}}/N_{\text{Fl,0}}}{\tau_{\text{rad,Fl}}} \]

Rewriting of Eq.\text{S1}1a gives

\[ \frac{\Delta N_{\text{F,b,p,\infty}}}{N_{\text{Fl,0}}} = \frac{\delta_{N_{\text{F,b,p,\infty}}}/N_{\text{Fl,0}}}{\tau_{\text{rad,Fl}}} \]

Insertion of numbers (\( \tau_{\text{rad,Fl}} = 115 \text{ ps} \) (see Fig.\text{S}5), \( \tau_{\text{rad,Fl}} = 19 \text{ ns} \) and \( \Delta \phi_{\text{F,da,\infty}} = 0.0101 \) gives \( \delta_{N_{\text{F,b,p,\infty}}}/N_{\text{Fl,0}} = 0.419 \). The fraction of flavin cofactor \( \delta_{N_{\text{F,b,p,\infty}}}/N_{\text{Fl,0}} \) is within experimental accuracy equal to the mole-fraction of FAD in the flavin composition (\( \chi_{\text{FAD}} = 0.396 \pm 0.015 \)).

The excitation wavelength dependence of the dark-adapted fluorescence quantum yield \( \phi_{\text{F,da}} \) after three photo-cycles is shown by the dotted-line connected triangles in Fig.\text{S}2.

Fluorescence quantum distributions of dark-adapted RmPAE after three photo-cycles for different fluorescence excitation wavelengths \( \lambda_{\text{exc}} \) in the range from 480 nm to 230 nm are displayed in Fig.\text{S}10. Compared to the fluorescence quantum distributions of fresh unexposed RmPAE (Fig.\text{S}1) the main difference is the increased flavin emission due to the release of proper non-covalently bound flavin in the RmPAE BLUF domain to improper non-covalently bound flavin. The emission around 450 nm is increased likely due to some photo-degradation of flavin to lumichrome. The emission around 400 nm is increased likely due to some photo-degradation of flavin to quinoxaline hydrolysis products.

### S6. Photo-degradation of RmPAE in the signaling state

In Fig.\text{S}11 the development of the attenuation coefficient spectrum of RmPAE due to light exposure at \( \lambda_{\text{exc}} = 455 \text{ nm} \) with \( I_{\text{exc}} = 0.108 \text{ W cm}^{-2} \) is shown. Accumulated exposed excitation energy densities \( w_{\text{exc}} \) are listed in the legend. The thick dotted curve belongs to the unexposed sample (sample in receptor state). The thin solid curve represents the sample in the signaling state (sample exposed with \( w_{\text{exc}} = 1.15 \text{ J cm}^{-2} \), negligible degradation in signaling state). The thick solid curve shows the attenuation coefficient spectrum at the end of the light exposure (accumulated exposed energy density \( w_{\text{exc}} = 666.16 \text{ J cm}^{-2} \)). The thick dash-dotted curve shows the attenuation coefficient spectrum of the sample which was heat denatured three days after the photo-degradation (sample inserted in heat bath at 66.3 °C and heated up to 85.6 °C within 10 min, then cooled down and centrifuged at 4 °C for 15 min with 4400 rpm).

The inset in Fig.\text{S}11 shows the absorption cross-section spectra of lumichrome in aqueous solution at pH 8 (from [22]) and of quinoxaline derivatives (from [23]).
In Fig.S12 fluorescence quantum distributions $E_p(\lambda)$ of RmPAE after photo-degradation (see Fig.S11) are shown in the range from $\lambda_{exc} = 480$ nm to 230 nm in steps of 10 nm. In Fig.S13 the fluorescence quantum distributions are shown after additional heat denaturing of RmPAE.

**S7. Structural formulae of some involved flavins:** Structural formulae of fully oxidized flavin $Fl_{ox}$ (flavin quinone Fl), lumichrome LC, (reduced) flavin – tyrosinyl adduct Fl-Tyr, and a quinoxaline derivative QO1 (1,2-dihydro-2-keto-1,6,7-trimethyl-quinoxaline-3-carboxylic acid) are shown in Fig.S14.

**Summary**

The pink to light reddish-pigmented bacterium *Rubellimicrobium mesophilum* strain MSL-20$^1$ contains a BLUF coupled endonuclease III (BLUF-EndoIII) of unknown function. This BLUF-EndoIII protein was identified through metagenome analysis by Conserved Domain Search in NCBI portal. A sequence alignment study of the BLUF domain of BLUF-EndoIII with the BLUF domain of AppA from *Rhodobacter sphaeroides* indicated that three essential flavin binding amino acids for BLUF activity were different. Therefore a purified recombinant triple mutated BLUF coupled endonuclease III (F5Y, N27H, A87W) named RmPAE (*Rubellimicrobium mesophilum* Photo-Activated Endonuclease) was expressed in *Escherichia coli*, and it was characterized by absorption and emission spectroscopic methods.

The flavin cofactor loading was determined by absorption spectrum analysis to be about 26 %. The flavin composition was deduced from fluorescence quantum yield measurement of heat denatured RmPAE to be approximately 60 % of FMN (and/or riboflavin) and 40 % FAD.

The primary BLUF domain photo-cycling behavior of RmPAE starting from its receptor state was similar to other BLUF proteins studied thus far. The first flavin absorption band spectral red-shift in the signaling state of about 5.4 nm was rather small, and the signaling state recovery to the receptor state after light switch-off of about 19 min at room temperature was rather long. Different to other BLUF proteins, for RmPAE in the signaling state, there occurred partial removal of BLUF active flavin in proper non-covalently bound position to BLUF inactive flavin in improper position (likely FAD cofactor re-localization).

The receptor-state signaling-state photo-cycling dynamics is presented in a refined photo-cycle scheme and a ground-state and excited-state reaction coordinate scheme. Energetic and kinetic parameters are extracted.

Photo-excitation of RmPAE in its light-adapted signaling state (secondary BLUF domain photo-cycling) caused low-efficient permanent covalent reduced flavin – Tyr adduct formation and conversion of flavin to lumichrome and quinoxaline derivatives.
References


18. Lindsey J, PhotochemCAD Spectra by Chemical Spectra Design, USA.


