Electronic supplementary information (ESI) for:

Single mutation in a novel bacterial LOV protein yields a singlet oxygen generator

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Content:

Materials and Methods

Cloning and protein expression. All genetic manipulations were conducted according to standard protocols. DNA encoding the gene *mrad2831_4511* (for protein *Mr*4511) was amplified by PCR from genomic DNA of *Methylobacterium radiotolerans* ATCC 27329/DSM 1819 with primers P1 and P2 (Table 1, Eurofins Genomics, Ebersberg -GER), then cloned into pET30 via restriction sites NdeI and XhoI (Fermentas, Waltham, MA-USA) for C-terminal fusion to a His_6-tag to facilitate protein purification. The mutants C71S, C71G, C71SQ112W and C71GQ112W were constructed using PCR with primers P3-P14 (Table 1) by site-directed mutagenesis. The ligation products were transformed into *Escherichia coli* BL21 (DE3) cells. All the *Mr*4511 protein variants were expressed by the following protocol: the transformed cells were cultured in DYT medium (16 g Trypton, 10 g yeast-extract, 5 g NaCl in 1 L distilled water) supplemented with kanamycin (20 μg mL^{-1}). After induction with isopropyl β-D-thiogalactoside, IPTG (1 mM final concentration) for 16 hours at 16℃, cells were harvested by centrifugation at 6500 rpm (Beckman Coulter, Avanti™ J-20 XP, JA-10) for 5 min at 4 ℃ and washed with distilled water for the next step. For protein purification, the cell pellets were resuspended in ice-cold KPB buffer (20 mM, pH 8.0) containing 0.3 M NaCl and disrupted by ultrasonic machine (0 °C, pulse on: 1 s, pulse off: 2 s, total duration time of 24 min; Nanjing Safer Biotech). The suspension was centrifuged at 15000 rpm (Beckman Coulter, Optima™ L-80 XP Ultracentrifuge, Ti-60) for 60 min at 4 ℃. The supernatants were purified via Ni^{2+}-affinity chromatography on chelating sepharose (GE healthcare). After elution from the Ni^{2+}-affinity column, imidazole was removed by dialysis against Na-phosphate buffer (10 mM, pH 8.0) containing NaCl (100 mM).

Table 1. Primers for the variants of *mrad2831_4511*

Primer	Sequence (5' to 3')	Plasmid
P1	AGGCTAGACATATGGAGACGGGCGGGACC	WT
P2	TAATATCTCGAGGCGGGCCGCGAGCTT	
P3	TCCCGCTTCCTGCAGGGG	C71S
P4	GTTGCGGCCGACCACCTCC	
P5	GGCCGCTTCCTGCAGGGG	C71G
P6	GTTGCGGCCGACCACCTCC	
P7	GAACGCCCTCTATGTCGG	C71SQ112W
P8	CAGAAGGTCGAGCCGTCC	
P9	GAACGCCCTCTATGTCGG	C71GQ112W
P10	CAGAAGGTCGAGCCGTCC	

Steady state spectroscopy. Absorption and fluorescence spectra were recorded with a Jasco 7850 UV/Vis spectrophotometer (Jasco Europe, Lecco, ITA) and with a Perkin Elmer LS50 luminescence spectrometer (Perkin Elmer, Waltham, MA-USA) respectively, both provided with thermostated sample holders. The luminescence spectrometer was equipped with polarizers for fluorescence anisotropy measurements. When required, the cuvettes were illuminated from above with a blue-light emitting Led-Lenser®V8 (7559) lamp (Zweibrüder Optoelectronics, Solingen-Germany) $\lambda_{em,max}$ = 462 nm (LED462), P_{max} = 8 mW.

Time-resolved absorption and fluorescence spectroscopy. For single-shot measurements of triplet-triplet absorption decays, excitation was performed using the third harmonic (355 nm) of a Q-switched Nd:YAG laser (Innolas, Krailing, Germany). Transient absorbance changes were monitored at 720 nm (maximum in the triplet absorption spectrum, Fig. S3), filtering the detection light (CW 75 W Xenon lamp, Amko) through two monochromators (master, slave). A photomultiplier coupled to a digital oscilloscope (LeCroy LT374, 500 MHz, 4 GS/s) provided the collection of the transmitted light. To improve signal to noise ratio, the recorded data were filtered by a 2-bit enhanced resolution function. Measurements of the transient bleaching of the parent state were detected at 450 nm, using excitation light at 475 nm from an OPO (GWU, Erftstadt, Germany) driven by the third harmonic of the Q-switched Nd:YAG laser. To improve signal to noise ratio, 9 laser pulses were averaged for each measurement and a 2-bit enhanced resolution was performed. Kinetics for triplet decay and recovery of parent state bleaching matched for all measurements. The maximum bleaching of the parent state was used to determine triplet formation quantum yields (Φ_T), considering the determined absorption coefficient values (see below) and taking FMN as a reference with a Φ_T = 0.6.[1]

All measurements were done at (22±1) °C using 1 cm light-path quartz cuvettes, with 0.4 cm width. In order to avoid filter effects, the sample absorbance at the detection wavelength did not exceed 0.6. The experiments were performed using a single shot of ca. 1 mJ in energy that was within the linear regime. The data were handled and analyzed using Origin Professional version 9.1 (Microcal Software, Inc., Northampton, MA, USA).

Fluorescence lifetimes were measured by a Time-Correlated Single-Photon Counting (TCSPC) approach using FLS920 (Edinburgh Instruments, UK). The sample excitation was achieved through a pulsed LED at 450 nm with 6 MHz repetition rate (PicoQuant, Berlin, Germany). The sample absorbance at the excitation wavelength did not exceed 0.1. The instrument response function was determined at the excitation wavelength using a very diluted solution of colloidal silica (LUDOX). In order to ensure that only one photon per light pulse was detected, the photon rate did not exceed 5% of the exciting light rate.

The equipment used to measure triplet-triplet absorption spectra have been described before.[2] The filtered output of a Xenon lamp (Oriel) was passed through the sample and into a spectrograph (Andor Shamrock 303). The spectrograph has two independent output ports; one equipped with a time-gated CCD camera (Andor iStar) to record transient absorption spectra and another equipped with a PMT (Hamamatsu

H10720) to record time-resolved absorption traces. Samples were excited at 420 nm using the frequency-doubled output of a femtosecond pulsed laser (Spectra-Physics Spitfire) operated at a repetition rate of 100 Hz.

Singlet oxygen (SO) quantum yield (Φ_Δ) determination

The equipment and procedure used to determine singlet oxygen quantum yields (Φ_Δ) by detecting the transient phosphorescence of SO at 1275 nm have been described elsewhere[3,4]. Samples were excited at 420 nm using the frequency-doubled output of a femtosecond pulsed laser (Spectra-Physics Spitfire) operating at a repetition rate between 1 kHz – 100 Hz. The characteristic phosphorescence from SO at 1275 nm was isolated with a 1064 nm long-pass and a 1270/20 nm band-pass filter in front of a NIR sensitive PMT (Hamamatsu R5509). Sulphonated Phenalenone (PNS) in H_2O or D_2O-based NaPi-buffer (ϕ_Δ = 0.97 ± 0.06)[4] served as a reference sensitizer.

For determination of Φ_Δ with with a fluorescence probe, Singlet Oxygen Sensor Green (SOSG) was purchased from Thermo Fisher Scientific (Waltham, MA-USA). The content of one vial of SOSG was dissolved in 33 µl of methanol to make a stock solution of ≈ 5 mM. Then a working solution of ≈ 10 µM was prepared in buffer immediately before use. Concentrations used for measurements were ≈ 2 µM of SOSG and ≈ 8 µM of protein/FMN in Napi buffer 10 mM, 100 mM NaCl, pH=8 or pD=8. Increasing in SOSG fluorescence was monitored at 530 nm with a Perkin Elmer LS50 luminescence spectrometer (Perkin Elmer, Waltham, MA-USA) operating in the kinetic mode, exciting the sample at 500 nm (to minimize FMN absorbance) and keeping the cuvette illuminated from above with LED462. The four mutated proteins were studied both in H_2O and D_2O NaPi buffer (pH =8 or pD = 8). FMN was used as a reference, with $\Phi_\Delta = 0.56 \pm 0.05$ and 0.64 ± 0.05 in H_2O and D_2O solutions respectively. [5] Control measurements were performed with: i) SOSG alone having the same concentration as in the sample containing SOSG and the photosensitizer (protein or FMN); ii) protein/FMN alone having the same concentration as in the sample containing SOSG and the photosensitizer; iii) adding 20-40 mM of the SO quencher azide (NaN_3) to the sample containing SOSG and the photosensitizer.

Determination of absorption coefficients.

Proteins from stock solutions in phosphate buffer were diluted in Guanidine hydrochloride (GuCl) 8 M (50 µl of protein stock solution/950µl of GuCl). Absorption and fluorescence spectra were measured at t = 0 and after 20 h; at t = 0 the proteins appeared intact, with spectra resembling those in phosphate buffer. At t = 20 h FMN was completely dissociated and the chromophore concentration was the same as in the intact protein. For control free FMN spectra were recorded in buffer and in GuCl, in both conditions FMN showed the same spectral features. Knowing the absorption coefficient of FMN at 445 nm (12500 M^{-1} s^{-1})[6] we calculated the values for protein-bound FMN (Table 1)

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AtPhot2-LOV2 386 IEKNFVISDP RLPDNPIIFA SDSFLELTEY SREEILGRNC RFLQGPETDQ
miniSOG        1 MEKSFVITDP RLPDNPIIFA SDGFLELTEY SREEILGRNG RFLQGPETDQ
SOPP           1 MEKSFVITDP RLPDNPIIFA SDGFLELTEY SREEILGRNG RFLQGPETDQ
SOPP2          1 MEKSFVITDP RLPDNPIIFA SDGFLELTEY SREEILGRNG RFLQGPETDQ
SOPP3          1 MEKSFVITDP RLPDNPIIFA SDGFLELTEY SREEILGRNG RFLQGPETDQ
Pp2FbFP-L30M  14 SNDGIVVAEQ EGNESIMIYV NPAFERLTGY CADDILYQDA RFLQGEDHDQ
DsFbFP        33 AEMSVVFSDP SQPDNPMIYV SDAFLVQTGY TLEEVLGRNA RFLQGPDTNP
DsFbFP_M49I   33 AEMSVVFSDP SQPDNPIIYV SDAFLVQTGY TLEEVLGRNA RFLQGPDTNP
Mr4511-C71X   32 TRMPMIITDP AQHDNPIVFV NDAFLKLTGY TRMEVVGRNX RFLQGPDTEA

AtPHOT2-LOV2 436 ATVQKIRDAI RDQREITVQL INYTKSGKKF WNLFHLQPMR DQKGELQYFI GVQLDG
miniSOG       51 ATVQKIRDAI RDQREITVQL INYTKSGKKF WNLLHLQPMR DQKGELQYFI GVQLDG
SOPP           1 ATVQKIRDAI RDQREITVQL INYTKSGKKF WNLLHLQPMR DQKGELQYFI GVLLDG
SOPP2          1 ATVQKIRDAI RDQREITVQL INYTKSGKKF LNLLHLQPMR DQKGELQYFI GVVLDG
SOPP3         51 ATVQKIRDAI RDQREITVQL INYTKSGKKF LNLLNLQPIR DQKGELQAFI GVVLDG
Pp2FbFP-L30M  64 PGIAIIREAI REGRPCCQVL RNYRKDGSLF WNELSITPVH NEADQLTYYI GIQRDV
DsFbFP        83 HAVEAIRQGL KAETRFTIDI LNYRKDGSAF VNRLRIRPIY DPEGNLMFFA GAQNPV
DsFbFP_M49I   83 HAVEAIRQGL KAETRFTIDI LNYRKDGSAF VNRLRIRPIY DPEGNLMFFA GAQNPV
Mr4511-C71X   82 AAVDRLRAAI RREEDIRVDL LNYRKDGSTF QNALYVGPVR DEAGRVVYFF ASQLDV
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Figure S1: aligned amino acid sequences of photosensitizing LOV domains and the LOV core of *Mr*4511-C71X (X = G or S). MiniSOG was derived from the LOV2 domain of *Arabidopsis thaliana* phototropin 2 (UniProt code: P93025) (*At*Phot2-LOV2) introducing six mutations (evidenced in green),[7] including the reactive cysteine (C426, that became G40 with miniSOG numbering). SOPP proteins were derived from miniSOG by rational design of further mutations.[8] Pp2FbFP has been engineered from the LOV protein Q88JB0 (UniProt) from *Pseudomonas putida* and used as a full length protein (151 aa), with two mutations;[9] *Ds*FbFP was derived from a LOV protein from *Dinoroseobacter shibae* (UniProt A8LP63, 133 aa) by mutation of the reactive cysteine (C72A), together with a further variant at position 49 (M49I, corresponding to L30 of Pp2FbFP); [10] note valine 113 in place of miniSOG tryptophan 81. *Mr*4511 (UniProt B1M516, 164 aa) becomes a SO photosensitizer with a single mutation C71X (X = G or S); note glutamine 112 in place of miniSOG tryptophan 81.

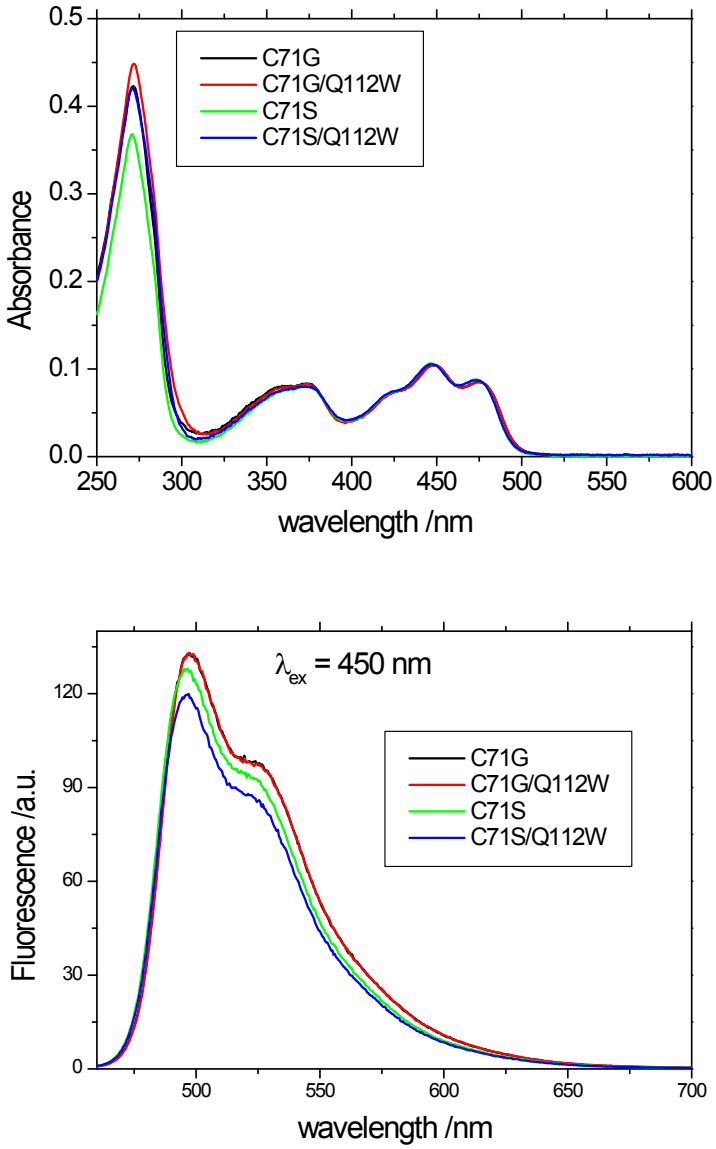


Figure S2: Absorption (top) and fluorescence (bottom) spectra (excitation at 450 nm, with matched absorbance at this wavelength, 20°C) of *Mr*4511 C71X (X = S or G) variants. Proteins solubilized in Na-phosphate buffer 10 mM, NaCl 100 mM, pH =8.

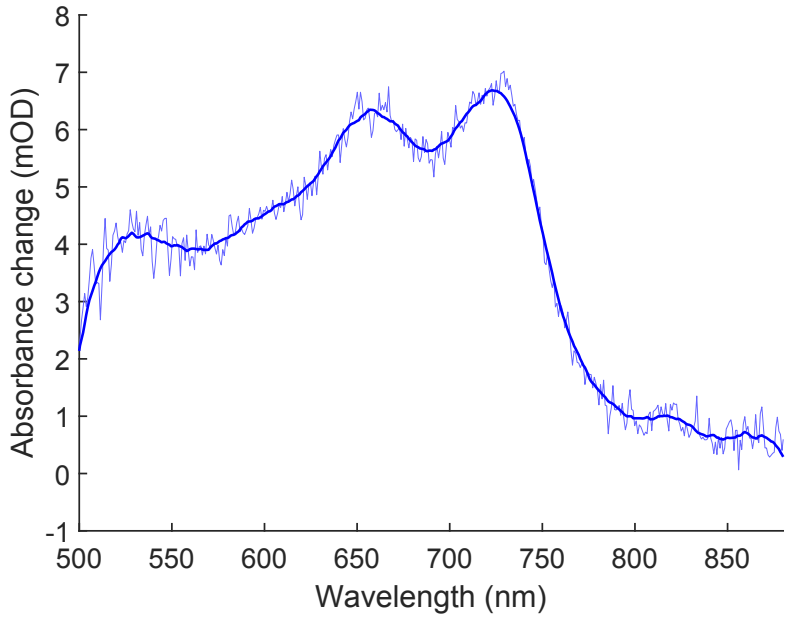


Fig. S3: Transient absorption spectrum of *Mr*4511-C71GQ/112W in air-saturated Na- phosphate buffered H_2O. The solid blue line is a numerical smoothing line meant only to guide the eye.

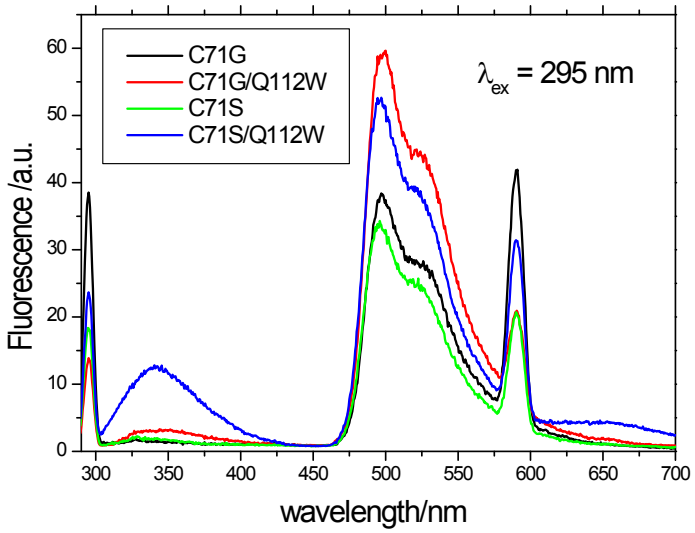


Figure S4: Fluorescence spectra of *Mr*4511 C71X (X = S or G) variants upon 295 nm excitation at 20°C. Proteins solubilized in Na-phosphate buffer; for all samples the absorbance for FMN at 448 nm was the same (0.1), which should ensure the same absorbance for FMN also at 295 nm (i.e. equal number of photons absorbed by FMN). Since the mutation at position 112 does not affect Φ_F of protein-bound FMN, the 55 % increase in FMN fluorescence intensity in the presence of W112 suggests that FRET (Foerster Resonance Energy transfer) occurs from W112 (when present) to FMN. Unfortunately, it is not possible to obtain a precise value for the absorbance or fluorescence (intensity or lifetime) of W112 in the absence of the acceptor (FMN), therefore the energy transfer efficiency could not be determined.

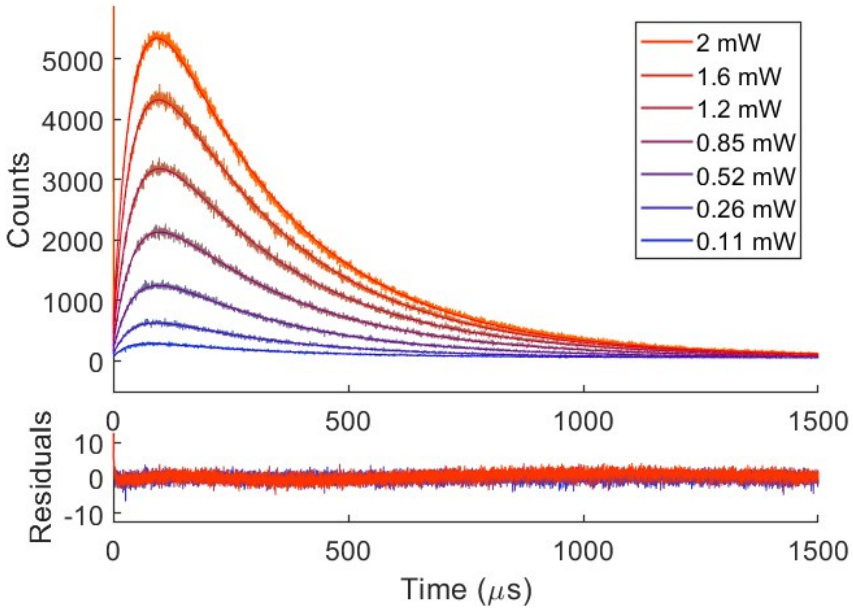
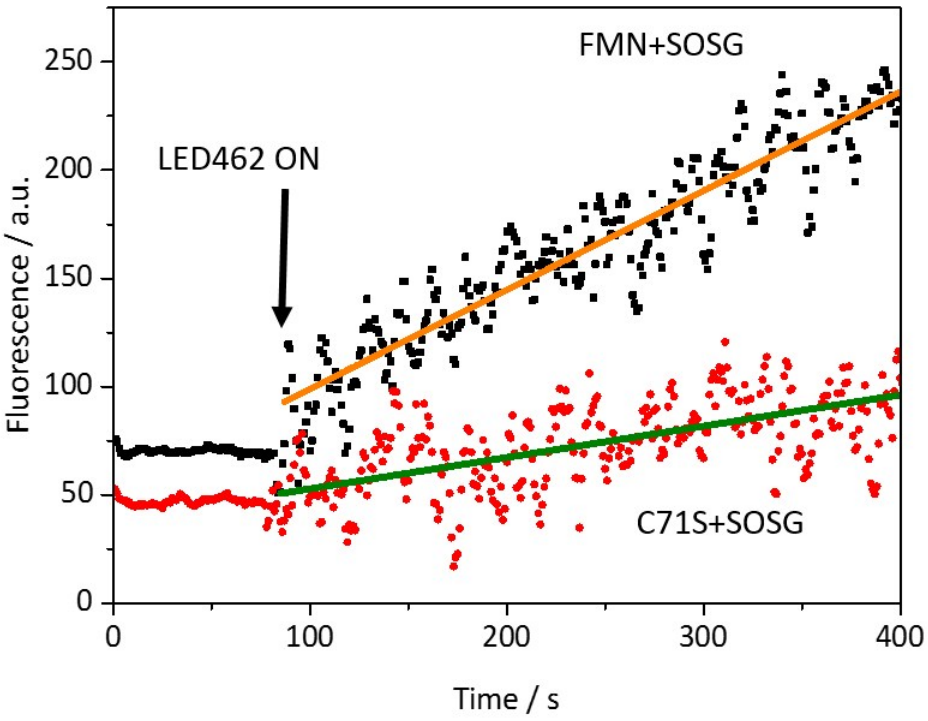


Fig. S5: Singlet oxygen phosphorescence traces from *Mr*4511-C71G in O_2-saturated Na-phosphate buffered D_2O as a function of the incident laser power. Fits to the data based on three exponential functions[4] are superimposed as solid lines on each kinetic trace. The intensity-normalized residuals to each fit are shown in the panel below.



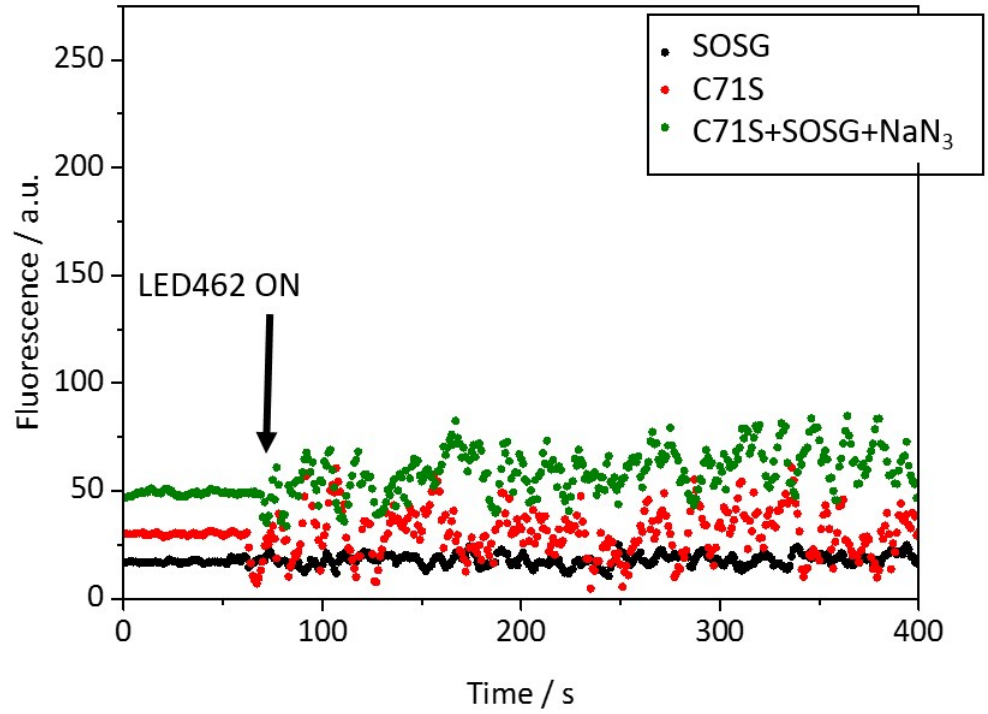


Figure S6: (top) Linear increase in the fluorescence of Singlet Oxygen Sensor Green (SOSG) at 530 nm (λ_{ex} = 500 nm) upon illumination with LED462. Linear fits are shown as solid lines. Φ_Δ for the protein was derived by comparing the slopes for the reference (FMN, Φ_Δ = 0.56 in these conditions, see Materials and Methods). (bottom) Control measurements showing that: a) in the absence of the photosensitizer, SOSG (dark) fluorescence at 530 nm increases negligibly; b) the protein alone shows a residual fluorescence at 530 nm that remains constant with LED462 illumination; c) in the presence of 40 mM azide (NaN_3), a quencher of SO, the increase of SOSG fluorescence is much lower. Measurements performed at 20°C, Na-phosphate buffer 10 mM, NaCl 100 mM, pH =8.

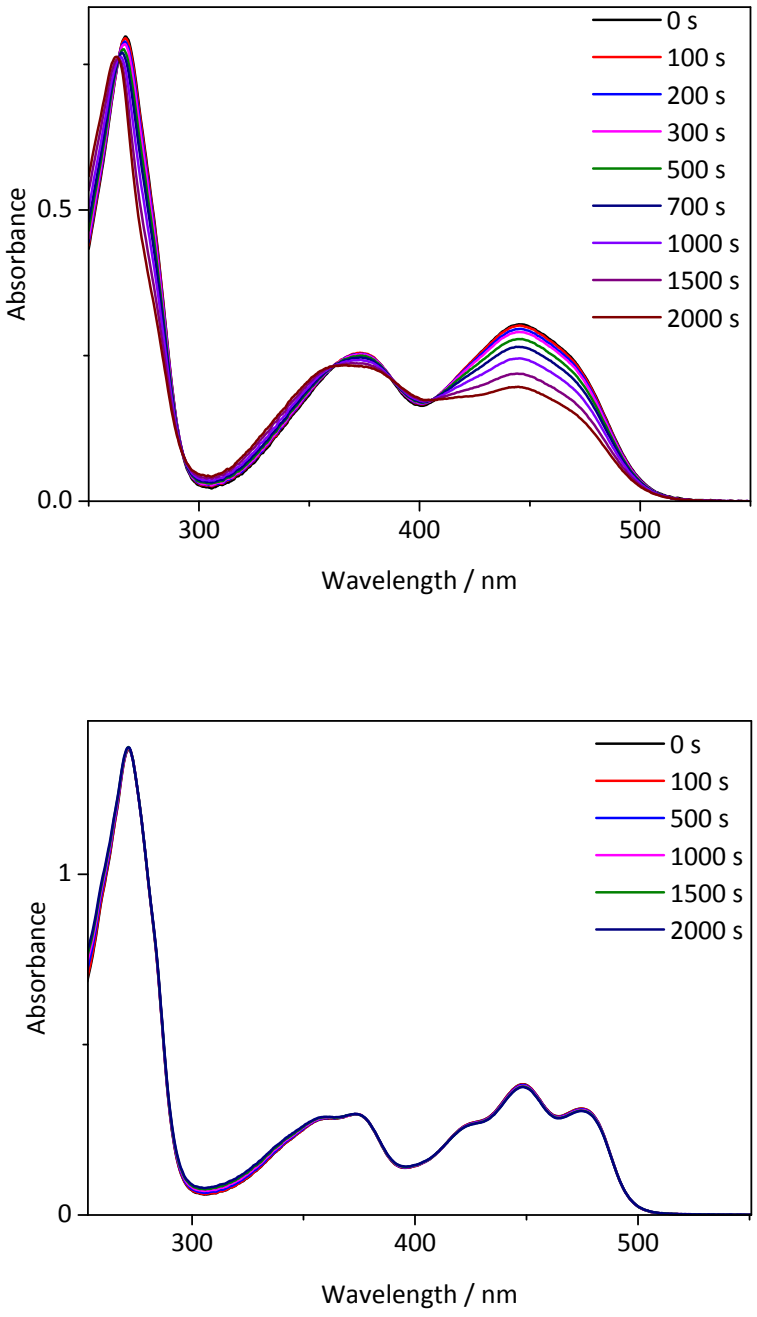


Figure S7: absorption spectra of FMN (top) and *Mr*4511 C71G (bottom) under prolonged illumination with LED 462 nm, from the top of the cuvette ensuring homogeneous illumination under our experimental conditions of very diluted solutions; most importantly the solutions were equally absorbing in blue-light region, ensuring that the same number of photons was absorbed. The measured LED 462 power was P ≈ 3 mW. The spectra were recorded after the illumination time steps shown in legend. Formation of lumichrome is seen for free FMN based on the gradual production of a species with a blue-shifted absorption maximum around 350 nm; [11]*Mr*4511 C71G has a very small evidence of degradation after prolonged illumination.

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