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

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***Mr*4511 from *Methylobacterium radiotolerans* is a 164 amino acid protein built of a flavin mononucleotide (FMN) binding, blue-light responsive LOV (Light, Oxygen, Voltage) core domain plus flanking regions. In contrast to the majority of LOV domains, *Mr*4511 lacks a tryptophan residue that was previously identified as a major quencher for the FMN triplet state in photosensitizers for singlet oxygen (SO) engineered from these photoreceptors. Here we show that for *Mr*4511 it is sufficient to only mutate the reactive cysteine responsible for the photocycle (Cys71) in the native protein to generate an efficient SO photosensitizer: both C71S and C71G variants exhibit SO quantum yields of formation, Φ_Δ, around 0.2 in air-saturated solutions. Under oxygen saturated conditions, Φ_Δ reaches ~0.5 in deuterated buffer. The introduction of Trp112 in the canonical position for LOV domains dramatically lowers Φ_Δ to values comparable to miniSOG, one of the early FMN binding proteins touted as a SO sensitizer. Besides its SO properties, *Mr*4511 is also exceedingly robust against denaturation with urea and is more photostable than free FMN.**

Methylobacterium radiotolerans is a γ-radiation resistant, pink colored plant symbiont and an opportunistic human pathogen belonging to α-proteobacteria.[1,2] Through genome digging,[3] researchers identified thirteen potential photoreceptors in this microorganism (data not shown): three bacteriophytochromes (bilin binding, red/far red sensors[4]), four BLUF (Blue Light sensing Using Flavins[5]) and six LOV (Light, Oxygen, Voltage[6]) (data not shown). LOV proteins are UVA/Blue Light (BL) sensors, preferentially binding flavin mononucleotide (FMN) as the chromophore. BL absorption triggers a photocycle that, in most cases, involves the reversible formation of a cysteine-FMN covalent adduct *via* the short μs decay of the FMN triplet state.[7] This photocycle prompted the development of LOV-based, genetically encoded photosensitizers for singlet oxygen (O_2 ($a^1\Delta_g$), or SO), the first of which was miniSOG (mini Singlet Oxygen Generator).[8] SO is an oxidizing excited state of O_2, commonly formed by photosensitization with triplet excited state chromophores.[9] SO has a variety of applications, from oxidative tagging in electron microscopy to cell ablation and optogenetics.[6] A prerequisite of a LOV-based SO photosensitizer is mutation of the reactive cysteine to prevent formation of the adduct and instead extend the lifetime of the flavin triplet state, plus other mutations aiming to minimize FMN triplet quenching by amino acids and increase O_2 accessibility to the chromophore cavity.[10–12] MiniSOG was derived from the LOV2 domain of a plant photoreceptor introducing six mutations (Fig. 1 and Fig. S1†).[8] The enhanced miniSOG variants, baptized SOPPs (Singlet Oxygen Photosensitizing Proteins), highlighted the importance of glutamine 103 and tryptophan 81 as major quenchers of the FMN triplet state.[13] Compared with the very low SO quantum yield of miniSOG, Φ_Δ = 0.03,[14,15] SOPP2 and SOPP3 showed an increase of up to 20-fold.[13] LOV-derived photosensitizers also emit fluorescence like other LOV-based flavin-binding fluorescent proteins (FbFP) used as reporters.[6]

The object of this present work is *Mr*4511, a 164 amino acid protein from *M. radiotolerans*, built of a standalone, photoactive LOV domain with short flanking regions. The position corresponding to W81, conserved in *ca.* 75% of LOV domains,[16] is occupied by Q112 (Fig. S1†). Based on the data obtained, the *Mr*4511-C71X (X = G or S) variants are proposed as novel, full length LOV-based SO photosensitizing proteins with a single mutation, enriching the existent toolbox[11] and exhibiting high stability. Cysteine 71 is the residue forming the adduct in the photocycle of the native protein (Consiglieri, unpublished results), corresponding to G40 in miniSOG numbering (Fig. 1 and Fig. S1†).

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AtPhot2-LOV2 386 IEKNFVISDP RLPDNPIIFA SDSFLELTEY SREEILGRNC RFLQGPETDQ ATVQKIRDAI RDQREITVQL INYTKSGKKF WNLFHLQPMR DQKGELQYFI GVQLDG
miniSOG        1 MEKSFVITDP RLPDNPIIFA SDGFLELTEY SREEILGRNG RFLQGPETDQ ATVQKIRDAI RDQREITVQL INYTKSGKKF WNLLHLQPMR DQKGELQYFI GVQLDG
SOPP           1 MEKSFVITDP RLPDNPIIFA SDGFLELTEY SREEILGRNG RFLQGPETDQ ATVQKIRDAI RDQREITVQL INYTKSGKKF WNLLHLQPMR DQKGELQYFI GVLLDG
SOPP2          1 MEKSFVITDP RLPDNPIIFA SDGFLELTEY SREEILGRNG RFLQGPETDQ ATVQKIRDAI RDQREITVQL INYTKSGKKF LNLLHLQPMR DQKGELQYFI GVVLDG
SOPP3          1 MEKSFVITDP RLPDNPIIFA SDGFLELTEY SREEILGRNG RFLQGPETDQ ATVQKIRDAI RDQREITVQL INYTKSGKKF LNLLNLQPTR DQKGELQAFI GVVLDG
Pp2FbFP-L30M  14 SNDGIVVAEQ EGNESIMIYV NPAFERLTGY CADDILYQDA RFLQGEDHDQ PGIAIIREAI REGRPCCQVL RNYRKDGSLF WNELSITPVH NEADQLTYYI GIQRDV
DsFbFP        33 AEMSVVFSDP SQPDNPMIYV SDAFLVQTGY TLEEVLGRNA RFLQGPDTNP HAVEAIRQGL KAETRFTIDI LNYRKDGSAF VNRLRIRPIY DPEGNLMFFA GAQNPV
Mr4511-C71X   32 TRMPMIITDP AQHDNPIVFV NDAFLKLTGY TRMEVVGRNX RFLQGPDTEA AAVDRLRAAI RREEDIRVDL LNYRKDGSTF QNALYVGPVR DEAGRVVYFF ASQLDV
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Fig. 1 Aligned amino acid sequences of photosensitizing LOV domains and the LOV core of *Mr*4511-C71X (X = G or S). The protein from which miniSOG was derived (the LOV2 domain of *Arabidopsis thaliana* phototropin 2, *As*Phot2-LOV2) is also shown to highlight the position of the reactive cysteine in wt LOV domains. For a more comprehensive description, see Fig. S1.†

The *Mr*4511 variants C71S and C71G exhibit spectral properties similar to other FbFPs (Table 1 and Fig. S2†),[17] with brightness ($\Phi_F \times \varepsilon$, where Φ_F = fluorescence quantum yield and ε = absorption coefficient in M^{-1} cm^{-1}) values that range from 4640 M^{-1} cm^{-1} to 5100 M^{-1} cm^{-1}, with negligible differences given our error limits.

Triplet lifetimes (τ_T) for the C71S and C71G variants are on the order of hundreds of μs (Table 2) and become longer after removal of oxygen by purging with N_2 for 90 min.

The triplet lifetimes we record are longer than those reported for free FMN under anaerobic conditions (~200 μs).[19] The latter could reflect self-quenching and possibly triplet–triplet annihilation,[20] phenomena strongly diminished for FMN within LOV proteins. It could also reflect the sensitivity of free FMN to residual dissolved oxygen in solution. Introducing a second mutation, Q112W, dramatically shortens τ_T (Table 2 and Fig. 2). We note that several biophysical techniques have identified radical pair formation *via* electron transfer from W112 to FMN chromophore in LOV domains devoid of the reactive cysteine.[21] Therefore, the same mechanism should be operating in Mr4511-C71X-Q112W variants. FRET experiments likewise indicate that W112 interacts with FMN (Fig. S4†). The distance between W112 and FMN is *ca.* 1.5 nm, according to crystal structures solved for LOV domains, a distance that is compatible with FRET experiments.[22,23] The relevance of tryptophan in this position in native LOV proteins nevertheless remains unclear, given that blue-light triggered photochemistry is not affected by its substitution. The FRET experiments suggest that this residue might serve as an energy donor to FMN upon UV-B irradiation.

The values of Φ_Δ were determined by time-resolved detection of SO phosphorescence at 1275 nm, as previously described,[13] and by using the fluorescent probe Singlet Oxygen Sensor Green(®) (SOSG) (Table 3, for details, see Materials and methods and Fig. S5 in the ESI†). The main reason to perform experiments in D_2O, as opposed to H_2O, is that the lifetime of SO is appreciably longer in D_2O (*ca.* 70 μs) than in H_2O (*ca.* 3.5 μs), thereby increasing the 1275 nm signal amplitude as well as facilitating discrimination between the kinetics of SO formation and decay.[9]

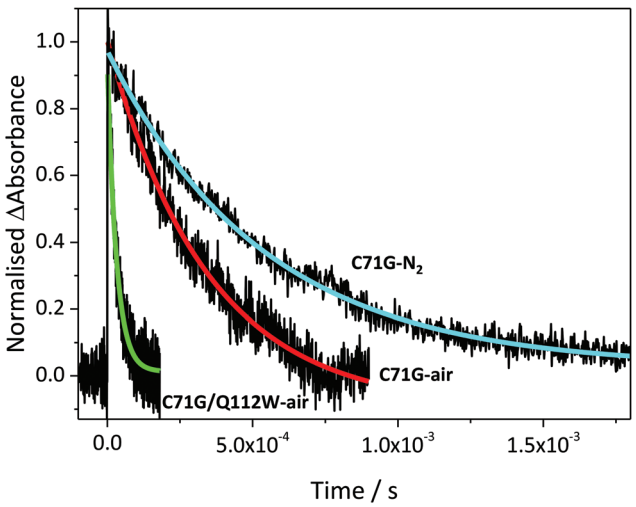

Fig. 2 Influence of tryptophan and deoxygenation on FMN triplet state decay (probed at 720 nm, excitation at 355 nm) for *Mr*4511-C71G, in single shot transient absorption experiments (H_2O_{buffer}). Fitted single-exponential functions are superimposed; see Table 2 for lifetimes and Fig. S3† for a transient absorption spectrum.

Table 1 Photophysical properties of *Mr*4511 variants[a]

*Mr*4511	ε_{448}/M^{-1} cm^{-1}	Φ_F	τ_S/ns	Φ_T
C71S	13 800	0.35	3.9	0.46
C71S/Q112W	14 500	0.32	3.8	0.50
C71G	13 800	0.37	4.5	0.58
C71G/Q112W	13 600	0.37	4.3	0.56

[a] see the ESI† for details; measurements in Na-phosphate buffer, 10 mM, pH 8, in NaCl 100 mM. Errors are ±10%.

Table 2 Triplet lifetimes (τ_T)

	H_2O_{buffer}, τ_T/ μs		D_2O_{buffer}, τ_T/ μs	
	Air	N_2	Air	N_2
*Mr*4511				
C71S[a]	240	362	446	549
C71S/Q112W[a]	24.7	27.3	29.7	29.8
C71G[a]	342	541	498	727
C71G/Q112W[a]	31.2	32.5	38.0	41.5
Others, 23 °C				
FMN[18]	2.94	—	3.07	—
MiniSOG[18]	31.3	33.6	38.6	41.8
MiniSOG-W81F[10]	—	—	265	1100
SOPP[18]	96.3	141	122	205
SOPP2[b]			130	1000
SOPP3[b]			135	3300

[a] From transient absorption experiments, excitation is at 355 nm, 22 °C. Errors are within 15%. [b] From Fig. S3 and Table 3 of ref. 13. Air = air saturated; N_2 = purged 90 min with nitrogen.

Table 3 Singlet oxygen quantum yields (Φ_Δ)

	Φ_Δ in H_2O_{buffer}		Φ_Δ in D_2O_{buffer}	
	Air	$+O_2$	Air	$+O_2$
*Mr*4511				
C71S	0.17[a]	0.23	0.20/0.26[a]	0.45
C71S/Q112W	—	0.04	0.01	0.03
C71G	0.19[a]	0.31	0.20/0.19[a]	0.45
C71G/Q112W	—	0.04	0.01	0.04
Others				
FMN[18]	0.56	0.58	0.64	0.68
MiniSOG	0.03 (ref. 14)		0.03 (ref. 13)	0.14 (ref. 13)
MiniSOG-W81F			0.33 (ref. 10)	
SOPP[24]	0.19		0.23	0.44
SOPP2[13]			0.51	0.55
SOPP3[13]			0.61	0.60
Pp2FbFP-L30M[11,25]	0.09 (ref. 25)		0.11 (ref. 11)	
DsFbFP[11]			0.33	

[a] Measured using SOSG, taking as reference FMN with Φ_Δ = 0.56 and 0.64 in aqueous and deuterated buffer, respectively (see Fig. S6† for an example).[18] All other values have been determined by time-resolved detection of SO phosphorescence at 1275 nm using sulphonated phenalenone (PNS) as reference (Φ_Δ = 0.97 ± 0.06).[26] Errors are ±15%. Measurements performed at 22 °C. Air = air saturated; O_2 = oxygen saturated.

The absence of W112 ensures Φ_Δ comparable to SOPP, while the introduction of a tryptophan in this position results in values comparable to miniSOG. A similar enhancing effect has been recently reported for the W81F variant of miniSOG[10] and for *Ds*FbFP (engineered from *Dinoroseobacter shibae*) that bears a valine instead of a tryptophan and has Φ_Δ = 0.33.[11] Another bacterial LOV protein tested as a SO photosensitizer was Pp2FbFP-L30M, bearing two mutations with respect to the native photoreceptor from *Pseudomonas putida*,[25] with Φ_Δ = *ca.* 0.11.[11] We note that for *Mr*4511-C71S, both τ_F and τ_T are shorter than for *Mr*4511-C71G, indicating that internal conversion from the FMN singlet excited state and intersystem crossing from the triplet state are faster in the former variant. To our knowledge, this effect of serine in place of glycine has not been reported before for any LOV-based SO photosensitizer.

Prolonged illumination of miniSOG induces the formation of photodegradation products:[14,15] FMN is gradually converted to lumichrome (LC) with consequent enhancement of SO production, and electron-rich amino acids become oxidized.[10] *Mr*4511 is more photostable than free FMN in our experiments (Fig. S7†), although this feature deserves further investigation *in vivo*.

A last and most astonishing feature of *Mr*4511 is its robustness against denaturation, a property that could be extremely important when using this protein as a fusion tag with *in vivo* applications. After 24 h in 7.8 M urea, C71S and C71G variants partially retain fluorescence anisotropy of the chromophore for many days (Fig. 3). For comparison, the LOV protein YtvA from *Bacillus subtilis*[27] is completely denatured after 30 minutes under the same conditions. *Mr*4511 is not as robust in 7.8 M guanidine hydrochloride (GuHCl), but it is still resistant enough to allow the determination of absorption coefficients (Table 1) in a simple and straightforward way (see Materials and methods in the ESI†).

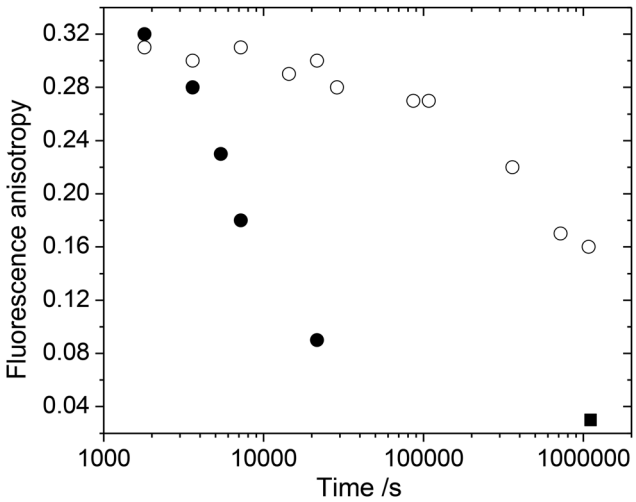

Fig. 3 Fluorescence anisotropy of FMN bound to *Mr*4511-C71 in 7.8 M urea (open circles) or GuHCl (filled circles); square: free FMN in 7.8 M urea. Excitation at 450 nm.

In conclusion, we present a novel, efficient, and robust genetically-encodable SO photosensitizer, adding to the available toolbox of LOV derived tools. Although the two variants C71S and C71G do not differ in their efficiency of SO production, at least *in vitro*, the FMN triplet and singlet state lifetimes are nevertheless affected by the changes in the surrounding protein. This may be important for their performance *in vivo*.

This novel bacterial LOV protein is the first photoreceptor characterized from the *Methylobacteria* group of pink pigmented facultative methylotrophic (PPFMs) bacteria that are mostly abundant in the plant microbiota,[28] and that are extremely rich in genes for photoreceptors.

Conflicts of interest

There are no conflicts to declare.

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