Reactive Oxygen Species in the Photochemistry of the Fluorescent Protein "KillerRed"

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Supporting information

1. Experimental details.

We synthesized the KRed gene, including a C-terminal his6-tag, via overlap extension PCR, cloned the gene into pProTet, and transformed the plasmid into *E. coli* BL 21. We overexpressed KRed to 20% of cell protein and purified the protein via Ni^{2+}-NTA-immobilized metal affinity chromatography (IMAC).

Irradiation for all experiments unless indicated otherwise was performed using a Hanovia carousel photoreactor equipped with a 450 W medium pressure mercury lamp. A 520 nm cutoff filter was used, so only the 546 nm and 578 nm Hg emission lines were utilized.

All experiments were performed in PBS aqueous buffer at pH 7.5 unless indicated otherwise. Ultraviolet-visible absorption spectra were recorded on a Perkin Elmer Lambda19 spectrophotometer. Measurements of photoluminescence were carried out using the Jobin-Yvon FluoroLog-3 spectrofluorimeter.

2. Singlet Oxygen Determination by direct spectroscopic method.

Singlet oxygen generation was detected by its characteristic phosphorescence at 1270 nm using a North Coast Scientific EO-817P germanium photodiode detector. A frequency-doubled Nd:YAG laser (Continuum Surelite I-10) was used as the excitation source providing 1–10 mJ per pump pulse at the sample at 532 nm with a pulse duration of around 10 ns. The standard $^1O_2$ photosensitizer tetrasulfonated porphyrin $TPPS^{4-}$ (ϕ_Δ = 0.7)[S1] was used to ensure that 1270 nm phosphorescence is readily detectable at these conditions. Figure S1 shows the experimental kinetic traces from $TPPS^{4-}$ and KRed measured in D_2O. Weak, but detectable signal was observed from the protein.

It is known that the phosphorescence spectrum of $^1O_2$ is quite narrow, and no appreciable signal at 1200 nm should be detected. Using the 1200 nm narrow band filter we performed the control experiments and compared the phosphorescence at 1270 nm to that at 1200 nm. This is a straightforward way to distinguish the $^1O_2$ emission from the triplet decay of the photosensitizer, which sometimes tails off to 1200 nm.

We have detected that the signal at 1200 nm for KRed is in fact several times more intense than at 1270 nm, and has exactly the same shape too (data not shown). Furthermore, the signal is reduced upon increase in the H_2O/D_2O ratio, repeating the same trend as seen at 1270 nm. Thus the signal at 1270 nm is simply the tail of the

[S1] Wilkinson, F.; Helman, W. P.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1995**, *24*, 663-1021.

more blue shifted signal, most likely the triplet state of KRed. Therefore, NO singlet oxygen can be detected from KRed in our direct experiment.

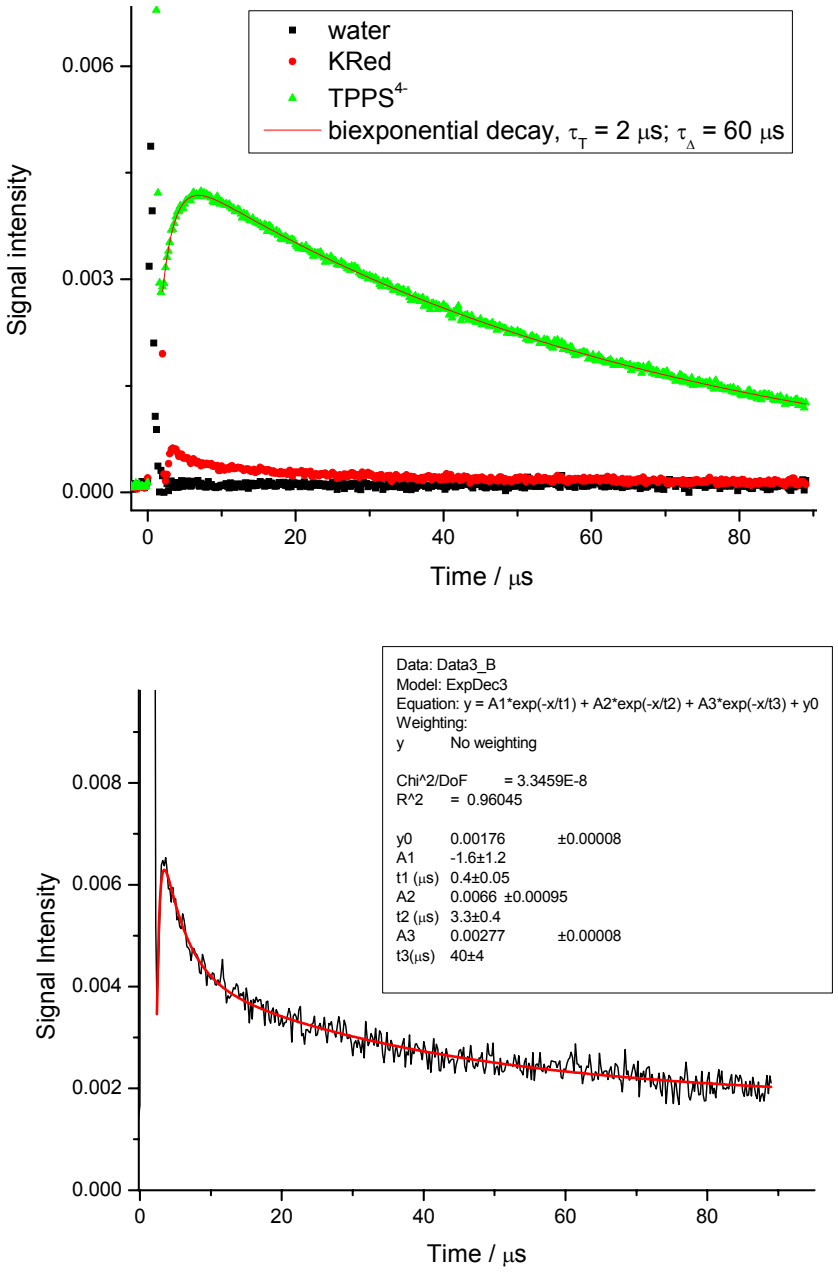

Figure S1. (Top) Time resolved traces recorded for pure D_2O, KRed and standard photosensitizer $TPPS^{4-}$ at 1270 nm following 532 nm excitation. $A_{532\ nm}$ = 0.1 for both dye solutions. (Bottom) Fitting of the KRed signal to 3-exponential model in PBS.

3. Oxygen Effects on Phototoxicity

KRed (13 μM) was bubbled with O_2 or N_2 for 15 min Absorbance and fluorescence spectra were taken before and after irradiation (Figure S2). In the presence of oxygen KRed bleaches considerably faster. In fact, in the presence of nitrogen, the very small bleaching can be attributed to the small amount of oxygen that is still present in solution. To examine effects of oxygen on the phototoxicity of KRed we irradiated 15 μM KRed with 20 μM of TEMPO-9ac in the presence and absence of oxygen (Figure S3). As expected, KRed generated more radicals in the presence of oxygen. Control containing 20 μM TEMPO-9ac in PBS irradiated under the same conditions displayed less than a 30% increase after 5 hrs of irradiation (data not shown).

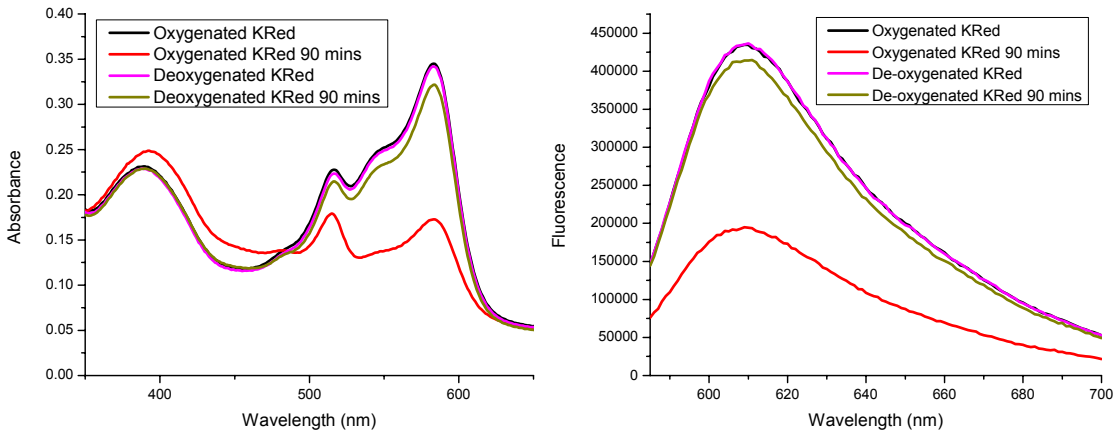

Figure S2: Absorbance (left) and emission (right, excitation 565 nm) spectra of KRed before and after 90 min irradiation and purged with either oxygen or nitrogen.

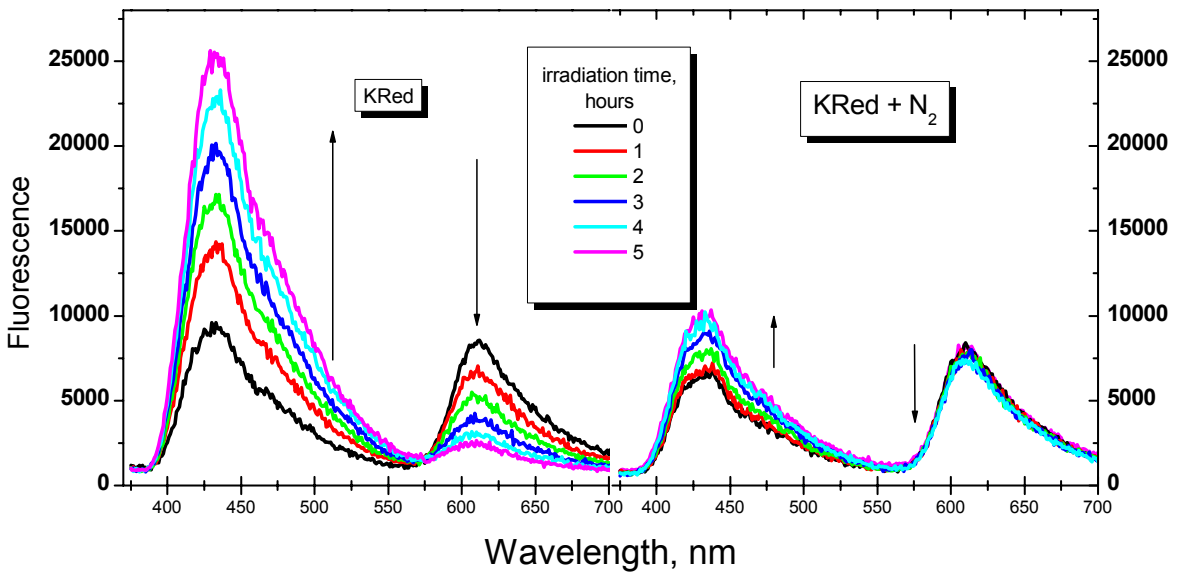

Figure S3: Fluorescence (λ_{ex}=358 nm) of KRed and TEMPO-9ac irradiated at 1 hr intervals. Left graph – the oxygenated sample, right graph – the sample bubbled with nitrogen.

4. EPR Studies

EPR spectra were obtained with a JEOL model FA-100 operating at X-band (9.39 GHz). The ESR spectrometer settings were: modulation frequency, 100 kHz; modulation amplitude, 3 G; microwave power, 1 mW; receiver gain, 3.99 x 10^6 and time

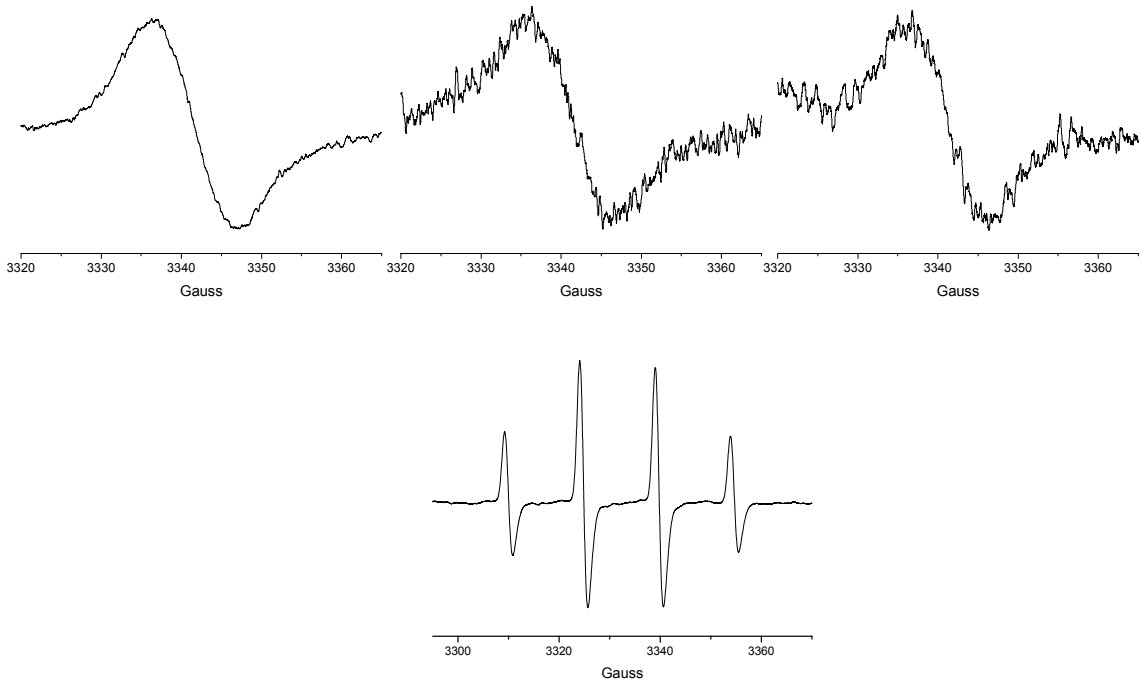

Figure S4: Top line, from left to right: EPR spectra of mRFP, DsRed, and EGFP obtained after irradiation. Bottom line: EPR spectrum of DMPO-OH adduct.[S2] OH radicals were generated by UV-decomposition of hydrogen peroxide.

constant 0.3 s.

5. Comparison of Radical Generation of KRed vs DsRed

Comparison of radical generation from KRed and DsRed was shown using TEMPO-9-ac. Separately KRed (8 μM) and DsRed (8 μM) were irradiated in the presence of 20 μM TEMPO-9-ac using a frequency-doubled Nd:YAG laser (λ=532 nm, 40 mW). Under identical conditions (power*time/abs) KRed generated a 10 fold increase in TEMPO-9-ac fluorescence over that of DsRed. A blank containing TEMPO-9-ac and PBS did not show any increase in fluorescence (data not shown).

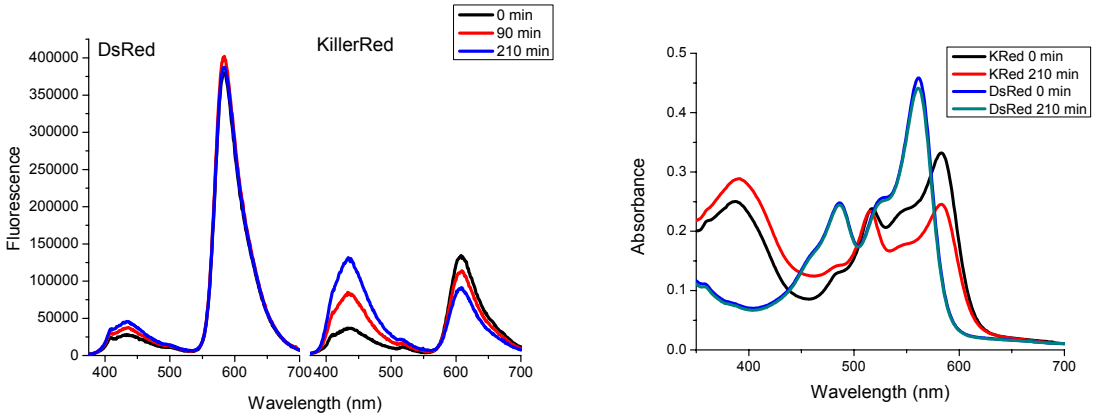

Figure S5: Emission spectra (λ_{ex}=358 nm) a) of Dsred (left) and KRed (right) with 20 μM TEMPO-9-ac and absorbance spectra b) of Kred and DsRed before and after irradiation.

6. Calibration Curve for Amplex Red Assay

Standard solutions of H_2O_2 were prepared and reacted with Amplex Red (Invitrogen, Carlsbad, CA) for 30 min in the dark.

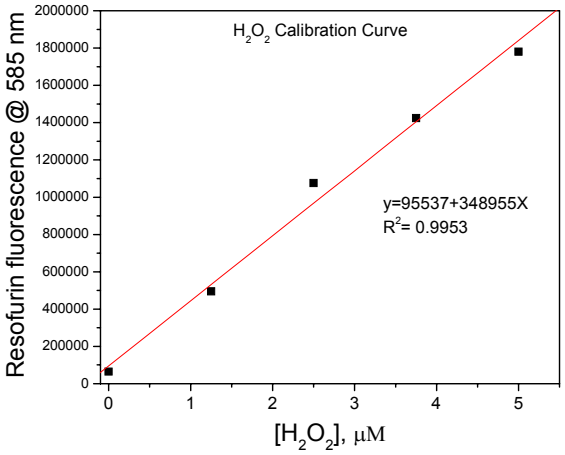

Figure S6: Calibration curve for Amplex Red Assay. Detection limit: 0.1 μM H_2O_2.

[S2] Souza, H. P.; Liu, X.; Samouilov, A.; Kuppusamy, P.; Laurindo, F. R. M.; Zweier, J. L. *Am J Physiol. Heart Circ. Physiol.* **2002**, *282*, H466-H474.

7. Bleaching of KRed with H_2O_2

Addition of 325 µM to 13 µM KRed resulted in 10% bleaching of 585 nm peak (Figure S7).

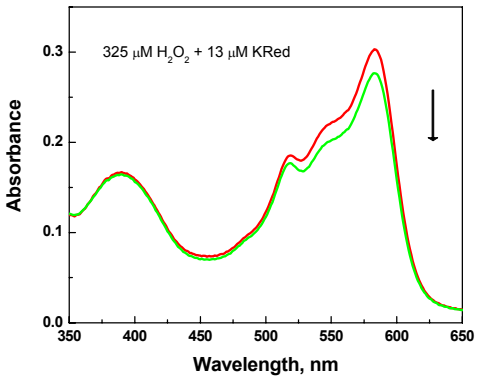

Figure S7: Bleaching of KRed upon addition of H_2O_2