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Temporal profile of the singlet oxygen emission endogenously produced by photosystem II reaction centre in an aqueous buffer.

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ABSTRACT

The temporal profile of the phosphorescence of singlet oxygen endogenously photosensitized by photosystem II (PSII) reaction centre (RC) in an aqueous buffer has been recorded using laser excitation and a near infrared photomultiplier tube. A weak emission signal was discernible, and could be fitted to the functional form $a\left[\exp\left(-t/\tau_2\right) - \exp\left(-t/\tau_1\right)\right]$, with $a > 0$ and $\tau_2 > \tau_1$. The value of τ_2 decreased from $11.6 \pm 0.5\,\mu s$ under aerobic conditions to $4.1 \pm 0.2\,\mu s$ in oxygen saturated samples, due to enhanced bimolecular quenching of the donor triplet by oxygen, whereas that of τ_1, identifiable with the lifetime of singlet oxygen, was close to 3 µs in both cases. Extrapolations based on the low amplitude of the emission signal of singlet oxygen formed by PSII RC in the aqueous buffer and the expected values of τ_1 and τ_2 in chloroplasts indicate that attempts to analyse the temporal profile of singlet oxygen in chloroplasts are unlikely to be rewarded with success without a significant advance in the sensitivity of the detection equipment.

Direct detection of singlet oxygen—molecular oxygen in its lowest excited electronic state (Schweitzer and Schmidt 2003)—becomes a herculean task when one wants to determine its concentration and temporal profile in a biological environment. The quantum yield of singlet oxygen phosphorescence (Φ_P) in water is extremely low, close to 5×10^{-7}, and the lifetime of singlet oxygen rather short, ~3.5 µs in neat water (Schmidt and Afshari 1990; Gorman and Rodgers 1992; Schweitzer and Schmidt 2003; Baier et al. 2005; Arellano et al. 2007a). Thorough analyses of the time-resolved phosphorescence emission of singlet oxygen in an aqueous environment became possible after the availability of NIR-sensitive photomultiplier tubes, which possess the requisite sensitivity and adequate time resolution (Jimenez-Banzo et al. 2008). The analysis of the temporal profile becomes even harder when singlet oxygen is produced by a photosensitizer dispersed in a biological (or a crowding) system, where biological compounds at high concentrations (*i.e.*, small cell metabolites, macromolecules or biological structures) dissolved in, or in close contact with, the aqueous medium provide additional pathways for the scavenging of singlet oxygen. Under these conditions, the lifetime of singlet oxygen (τ_Δ) is shortened to a range of only a few-hundred of ns (Gorman and Rodgers 1992; Bisby et al. 1999; Redmond and Kochevar 2006) and Φ_P decreases by at least 1–2 orders in magnitude in comparison with its value in water. The extremely low values for Φ_P and τ_Δ impose severe demands on the detection system, which must be sensitive as well as fast if one wants to analyse the temporal profile of singlet oxygen in a biological medium. Ogilby and co-workers (da Silva et al. 2012) have recently reported the temporal profile of singlet oxygen in HeLa cells after exchanging the intracellular water with deuterium oxide and they anticipate that the determination of τ_Δ and τ_D (the quenched lifetime of the donor triplet) can be a challenging and error-prone endeavour when the two lifetimes are very similar.

A satisfactory system for studying the kinetics of singlet oxygen emission should possess, apart from high sensitivity and rapid response, the capacity to discriminate effectively against extraneous luminescence. The singlet oxygen signal at 1270 nm is contaminated by phosphorescence and delayed fluorescence emissions from other molecules and scattered laser light. The intensity of the additional radiation can be several orders of magnitude larger than that of the singlet oxygen emission itself. Although the bulk of this unwanted radiation disappears in the first few hundreds of ns after the pulse excitation, its contribution must be determined in a control experiment (where the singlet oxygen emission is suppressed) and subtracted from the total signal from the sample under investigation. If the burst of stray light is sufficiently intense, it will drive the detector into a nonlinear region or even into

saturation, in which case the result of the subtraction will not be completely satisfactory, the early part of the subtracted signal will be devoid of significance, and a bi-exponential fitting of the temporal profile of the singlet oxygen emission will become prone to a large uncertainty, particularly when the rise time of the singlet oxygen emission is a few μs or shorter.

The kinetic traces satisfy the equation $I(t) = a\left[\exp(-t/\tau_2) - \exp(-t/\tau_1)\right]$, with $a > 0$ and $\tau_2 > \tau_1$; the shorter lifetime determines the rise time of the curve. One of the two lifetimes is to be identified with τ_Δ and the other with τ_D. If one compares two samples, which differ only in the concentration of dissolved oxygen, one of the two lifetimes should remain unchanged whereas the other would be shorter in the sample with the large concentration of oxygen.

An *in vitro* study where τ_2 has been demonstrated to depend on the concentration of molecular oxygen, has recently been published (Jarvi et al. 2011); on account of the difficulties mentioned above, they were not able to disentangle the early part of the curve, characterised by τ_1. Since most experiments designed to detect singlet oxygen in water or biological systems will suffer from similar handicaps, we have investigated a case (so far unique) in which singlet oxygen is endogenously photosensitized by a biological complex, namely the (type II) reaction centre (RC) of photosystem II (PSII), to see if it is possible to determine the formation and deactivation rate constants of the singlet oxygen produced by this complex in water. This is an intermediate situation between two extremes: a system where τ_Δ is significantly larger than τ_D, and the opposite case involving a system *in situ* (*i.e.*, plant chloroplasts or cells of oxygenic photosynthetic prokaryotes), where τ_Δ becomes *appreciably* smaller than τ_D. The feasibility of investigating the first case ($\tau_\Delta > \tau_D$) has already been established by Barber and co-workers (Macpherson et al. 1993; Telfer et al. 1999), who worked with PSII RC in deuterium oxide, and will not be further discussed in this technical communication. However, no results pertaining to the opposite inequality ($\tau_\Delta < \tau_D$) have been reported so far. Our results might shed some light on whether or not the analysis of the intensity and kinetic traces at 1270 nm of singlet oxygen produced by PSII RC *in situ* is a feasible task. The PSII RC consists of the heterodimer of the so-called D1 and D2 proteins, the cytochrome b_{559} subunits PsbE and PsbF, and the small subunit named PsbI (Barber et al. 1987; Nanba and Satoh 1987). The protein complex of PSII RC in its most active and stable form houses six chlorophyll (Chl) molecules, two pheophytin molecules and two β-carotene (β-Car) molecules. The pigment organization in the PSII RC complex, dictated by the protein matrix (Loll et al. 2005), is

unfavourable for efficient triplet-triplet energy transfer from ^{3}P to β-Car because the β-Car molecules are far from the accessory Chl in the D1 protein (Chl$_{D1}$), where the triplet is mainly localized and only a minor population is in the primary donor P$_{680}$ (Noguchi 2002). Hereafter, ^{3}P represents the sum of Chl$_{D1}$ and P$_{680}$ triplets in thermal equilibrium formed, through the radical pair mechanism (Takahashi et al. 1987), when the electron transport chain is inhibited on the acceptor side of PSII RC. The lifetime of the ^{3}P has been determined to be 600–1000 μs under anaerobic conditions, 20–40 μs under aerobic conditions and 4–6 μs under oxygenic conditions (Mathis et al. 1989; Telfer et al. 1999), which clearly indicates that ^{3}P is efficiently quenched by molecular oxygen. Evidence for triplet-triplet energy transfer from ^{3}P to molecular oxygen and the concomitant singlet oxygen production have been demonstrated in deuterium oxide (Macpherson et al. 1993; Telfer et al. 1994; Telfer et al. 1999).

The five-Chl PSII RC (RC5) was first isolated by Vacha and co-authors (Vacha et al. 1995) and minor modifications for its isolation procedure were introduced by our group (Arellano et al. 2007b). This type of RC5 is characterized by the loss of the peripheral Chl (Chlz) and β-Car molecules in the D2 protein during the isolation procedure and has been successfully used to monitor endogenous production of singlet oxygen in deuterium oxide (Arellano et al. 2011). The experimental procedure and the details of the experimental setup to follow the kinetic traces of the singlet oxygen emission have been described previously (Arellano et al. 2007a; Arellano et al. 2011). Aliquots of the stock solution of RC5 (dissolved in 0.01% (w/v) *n*-dodecyl *β*-D-maltoside, 50 mM Tris-HCl pH 7.2) were diluted in 20 mM Tris-HCl pH 7.2 to reach a final absorbance (for a path length of 1 cm) of 1.5 at 675 nm. RC5 samples were replaced with fresh samples every 256 scans before any significant change in absorption spectrum of RC5 could be identified. The cuvette with the RC5 sample was left open for experiments under aerobic conditions in equilibrium with the air atmosphere; whereas a constant stream of pure molecular oxygen blowing on the surface of the RC5 sample was used to ensure the oxygen saturation of the RC5 solutions inside the cuvette. Before the laser beam excitation, a volume of 20–30 mL of RC5 was also exposed to a continuous a stream of pure oxygen and incubated for approximately 1.0–1.5 h in the dark to ensure oxygen saturation. Aliquots of 1 mL of the RC5 sample were transferred from the reservoir to the cuvette with the aid of a long needle syringe to avoid any contact with the air of the room. The ratio of the oxygen concentration in aerated and oxygenated buffer was measured using a Foxy fiber optic oxygen sensor probe (Ocean Optics Inc, Dunedin FLA).

The temporal profile of the 1270 nm emission signal of singlet oxygen photosensitized by RC5 in 20 mM Tris-HCl pH 7.2 is depicted in **Fig. 1**. Although the kinetic traces show a poor signal-to-noise ratio, a time-dependent emission signal was discernible. The intensity of the signal was oxygen dependent and disappeared when oxygen was displaced with a stream of N_2 or NaN_3 was present in the medium (data not shown). A simple bi-exponential fitting of the temporal profile of the singlet oxygen emission yields two lifetimes with values close to $2.9 \pm 0.2 \, \mu s$ and $11.6 \pm 0.5 \mu s$ for τ_1 and τ_2, respectively. The estimation of the uncertainties of the two lifetimes is given in Online Resource 1. The latter value cannot be identified with τ_Δ, for it differs substantially even from the weighted lifetime of singlet oxygen in aqueous solutions containing a small percentage of detergent micelles. In these solutions, singlet oxygen is partitioned between the water phase and the detergent micelles, where the lifetime is longer because of the more hydrophobic environment (Lee and Rodgers 1983; Ohara et al. 2009; Arellano et al. 2011). The value of τ_1 cannot be identified with τ_D, for it is much shorter than the lifetime of ^3P under aerobic conditions, where values in the range of 20–40 μs have been reported depending on the temperature and the viscosity of the solution (Mathis et al. 1989; Telfer et al. 1999). The values for the ^3P lifetime found correspondence with the rise time of singlet oxygen when the PSII RC samples are dispersed in deuterium oxide, although these values decreased by approximately a factor of two when the energy power of the laser excitation beam was increased (Telfer et al. 1999). It follows therefore that we should identify τ_1 with τ_Δ and τ_2 with τ_D. In one of our previous studies, singlet oxygen emission in purple bacteria (type II) RC preparations was observed (Arellano et al. 2007a) and we concluded that the singlet oxygen produced by the RC was more likely to be deactivated by the solvent or the same RC where it was produced (geminate quenching) than by a different RC (non-geminate quenching). Based on the fact that both the PSII RC and the purple bacteria RC are very bulky protein complexes, it is reasonable to speculate that geminate quenching also takes place in PSII RC preparations and therefore a noticeable decrease in the value for τ_Δ must occur.

To check that our assignment was correct, we investigated the dependence of the two lifetimes on the concentration of dissolved oxygen in the sample. The temporal profile of the emission signal of the endogenous singlet oxygen produced by RC5 in the oxygen-saturated solution is depicted in **Fig. 1**. The peak height of the signal showed a nearly twofold increase and the signal-to-noise ratio improved notably. A bi-exponential fitting of the kinetic trace revealed two lifetimes with values 3.1 ± 0.2 μs and

4.1±0.2 µs for τ_1 and τ_2, allowing us to identify τ_2 with τ_D. We also measured the concentration of dissolved oxygen in oxygenated buffer and found it to be 3.23±0.03 times that in aerated buffer. The ratio of the values of τ_D in aerated and oxygenated buffer is 2.83±0.07. Though the discrepancy between the two ratios is outside the bounds of error, we feel that it is not serious enough to vitiate our principal conclusions.

In the exponential equation $a = C\tau_2 / (\tau_2 - \tau_1)$ and $C = N\sigma[\mathrm{P}]\Phi_\Delta / \tau_R$, where N stands for the number of incident photons per unit area, σ is the photosensitizer molecular absorption cross-section, [P] is the concentration of RC5, τ_R is the radiative lifetime of singlet oxygen in the medium and Φ_Δ is the quantum yield of singlet oxygen. Based on the fact that the decay rate of $^3\mathrm{P}$ in the presence of oxygen (either under aerobic or oxygenic conditions) is far larger than anaerobic conditions, the value for Φ_Δ can be considered nearly equal in aerobic and oxygenic conditions and, consequently, so can C. This implies that the integrated area of the emission signal of singlet oxygen should be similar in both experiments and that the maximum of its signal will depend on the formation rate constant of the singlet oxygen (i.e., τ_2 in this study). A theoretical simulation of the temporal profile of the singlet oxygen emission shows that the experimentally-observed changes in the profile from aerobic to oxygenic conditions follow the expected behaviour (Online resource 2).

The value of τ_Δ in cells, a matter of debate over the last two decades, has been reported to be lower than 1 µs in several studies (Gorman and Rodgers 1992; Bisby et al. 1999; Redmond and Kochevar 2006), and close to 3 µs in nuclei of neurons, where DNA was claimed not to be a particularly good quencher (Skovsen et al. 2005). An accurate value for τ_Δ in chloroplasts has not been reported yet, but based on the high concentration of soluble and membrane proteins, polyunsaturated lipids in thylakoids, and hydrophilic and lipophilic singlet oxygen scavengers (*i.e.*, carotenoid, vitamins E and C and glutathione), it is very unlikely that the value of τ_Δ can be larger than 0.5 µs. Based simply on the concentration of ascorbate in the stroma of chloroplasts (20–300 mM) (Smirnoff 2000), one can estimate a value for τ_Δ below 200 ns (Bisby et al. 1999). This value of τ_Δ can be underestimated if intracellular phenomena (e.g., molecular crowding and protein-protein interaction) are taken into account (Jensen et al. 2011). If $^3\mathrm{P}$ has a lifetime of 10–20 µs and τ_Δ in chloroplasts is as large as 0.5 µs (a rather optimistic value), the singlet oxygen should suffer a ten-fold decrease in intensity in comparison with that observed in water (Online resource 2). This estimate of the drastic reduction in the phosphorescence intensity, and

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the inevitability of interference from stray light during the rising part of the phosphorescence signal, suggest that the experimental determination of the kinetic rate constants of the singlet oxygen emission endogenously produced by PSII in chloroplasts would be a tall order indeed.

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Figure Legends

Fig. 1. Temporal profile of the emission signal at 1270 nm of singlet oxygen produced endogenously by RC5 in 20 mM Tris-HCl pH 7.2 under aerobic (black line) and oxygenic (red line) conditions. The absorbance of RC5 (for a path length of 1 cm) is 1.5 at 675 nm. The discontinuity in the y-axis illustrates the intensity of additional radiation reaching the NIR-sensitive photomultiplier tube. The number of averaged scans is 6144 and 5120 for aerobic and oxygenic conditions, respectively. The best fit for each signal and the residuals are also shown
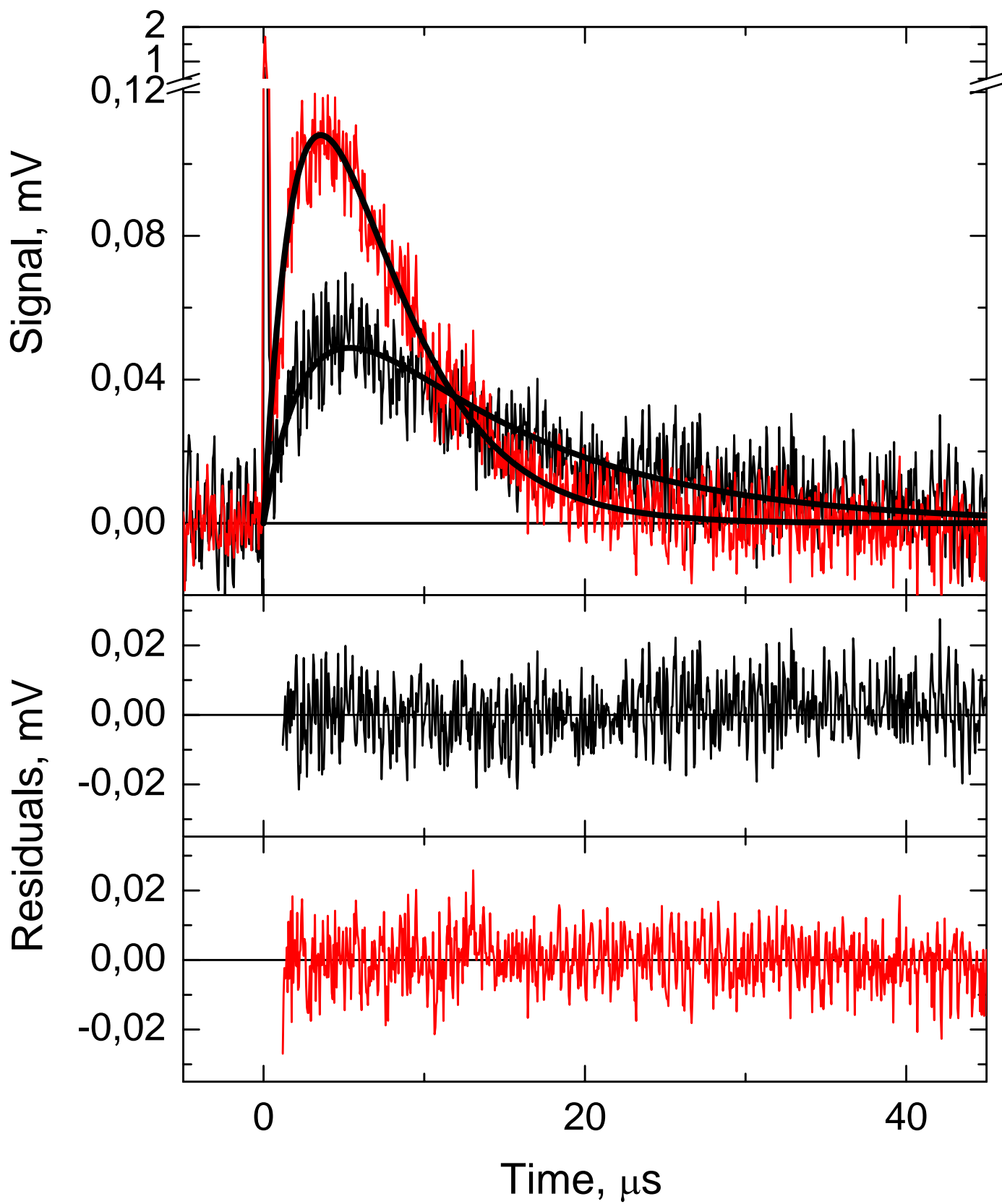
Photosynthesis Research

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centre in an aqueous buffer.

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Online Resource 1

Since we are not aware of any unambiguous and robust method of estimating the uncertainties of τ_1, and

τ_2, we have adopted the following approach. Three different softwares were tried for performing

constrained non-linear least-squares fittings: Matlab version 7.8 (Mathworks, Natick MA), OriginPro

version 8 SR4 (OriginLab Corporation, Northampton, MA), and the Solver tool in Microsoft Excel 2003

(Microsoft Corporate, Redmond WA). When reasonable values of the three adjustable parameters (a, τ_1,

and τ_2) became available, five (or more) new rounds of fitting were performed (by using Solver) in

which the initial guesses for the starting parameters were varied systematically, each round resulting in a

new set of best-fit values for the adjustable parameters. A fit was accepted only if the residuals were

found (on visual examination) to be uniformly distributed and the sum of squared residuals was within a

small margin (10%) of the values obtained in earlier rounds. Finally, the mean value of a parameter and

its standard error were calculated from the resulting data; the error quoted here is twice the standard error

so obtained. Once a set of values became available, a set of simulated signals were generated and

analysed by the three softwares to check that the retrieved values were in agreement with the values

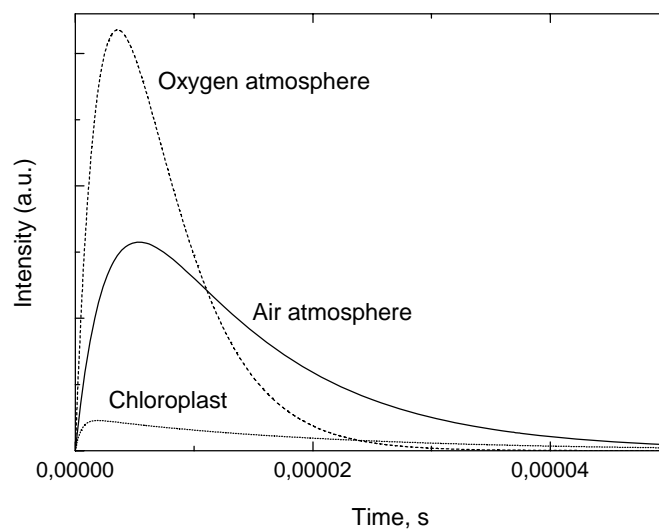reported here (and used for generating the simulated curves).

Figure Caption. Simulation of the temporal profile of singlet oxygen emission produced endogenously by RC5 using the rate constants derived from the bi-exponential fitting analysis. The noise-free temporal profiles are compared with that expected in chloroplasts assuming values of 0.5 µs and 20 µs for τ_{Δ} and τ_D, respectively