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Does Rose Bengal Triplet Generate Superoxide Anion?

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Rose bengal (RB) is well-established as an important photosensitizer for oxidation reactions and has been used widely in biology and chemistry.[1−4] The quantum yield for singlet oxygen [$O_2(^1\Delta_g)$] formation by energy transfer from the RB triplet state ($^3RB^*$) is high in water (0.75,[5] 0.8[6]) and in organic solvents (e.g., 0.76 in methanol[7]). Photosensitization by RB is frequently attributed exclusively to generation of $O_2(^1\Delta_g)$.[3,4] Quenching of $^3RB^*$ by oxygen has also been reported to generate superoxide ($O_2^{\bullet-}$) with a significant quantum yield ($\phi_{so} \approx 0.2$[8,9]) according to reaction 1. Superoxide anion formed *via* electron transfer from $^3RB^*$ has been considered as a reactive species for oxidations.[1−3,10−12]

$$^3RB^* + O_2 \rightarrow RB^{\bullet+} + O_2^{\bullet-} \qquad (1)$$

Recently, a study of energy transfer from $^3RB^*$ in reverse micelles to oxygen, which employed photoacoustic detection, indicated that ϕ_{so} was less than 0.1.[6] The previous studies[8,9] that gave higher values of ϕ_{so} employed indirect chemical methods to quantitate $O_2^{\bullet-}$. Because of the importance of RB-photosensitized oxidation in aqueous solution, the present work was carried out to resolve this inconsistency in the literature. Laser flash photolysis was employed to examine the indirect chemical methods used previously.[8,9]

The method employed by Srinivasan *et al.*[8] to quantitate $O_2^{\bullet-}$ formation by reaction 1 was based on oxygen consumption by the following chain reaction:[13]

$$O_2^{\bullet-} + H^+ \rightarrow HO_2^{\bullet} \qquad (2)$$

$$HO_2^{\bullet} + SO_3^{2-} \rightarrow HO_2^- + SO_3^{\bullet-} \qquad (3)$$

$$SO_3^{\bullet-} + O_2 \rightarrow SO_3 + O_2^{\bullet-} \qquad (4)$$

The influence of superoxide dismutase and 1,4-diazabicyclo[2.2.2]octane on the consumption of oxygen was used to deduce that the ratio of $O_2^{\bullet-}$ to $O_2(^1\Delta_g)$ produced by $^3RB^*$ was about 1:3 under the reaction conditions (32 mM Na_2SO_3, 69 μM RB, O_2-saturated pH 7.8 buffered aqueous solution). This method of monitoring $O_2^{\bullet-}$ assumes that reaction 5 is much slower than reaction 1. Sulfite has been previously reported to undergo electron transfer with carbonyl triplet states.[14] Quenching of $^3RB^*$ by electron donors has also been reported previously.[1,15] If reaction 5 occurs, $RB^{\bullet-}$ and $SO_3^{\bullet-}$ would transfer an electron to O_2 to generate $O_2^{\bullet-}$.[16,17]

$$^3RB^* + SO_3^{2-} \xrightarrow{k_{SO_3}} RB^{\bullet-} + SO_3^{\bullet-} \qquad (5)$$

In the current work the rate constant for reaction 5 was determined with the laser flash photolysis apparatus described previously.[18] $^3RB^*$ was generated using a 532 nm pulse (8 ns) from a frequency-doubled Nd:YAG laser. Typical laser energies were 2 mJ, and the laser beam was expanded to completely overlap the monitored volume. $^3RB^*$ was monitored at 620 nm, while the semioxidized ($RB^{\bullet+}$) and semireduced ($RB^{\bullet-}$) radicals were monitored at 470 and 420 nm, respectively.[17]

From a plot of the rate constant for $^3RB^*$ decay (k') versus [Na_2SO_3], k_{SO_3} was determined by linear regression to be 1.1 (±0.2) × 10^7 M^{-1} s^{-1}.[19] The time-resolved spectra in Figure 1 show the growth of absorption at 420 nm in agreement with the assignment of this band to $RB^{\bullet-}$ absorption. The decay of $^3RB^*$ is essentially complete (Figure 1, inset) at the longest time shown. The rate constant for the growth of $RB^{\bullet-}$ is similar to the decay of $^3RB^*$ monitored at 620 nm. The fraction of $^3RB^*$ quenched by sulfite under the conditions used by Srinivasan *et al.*[8] is given by

$$\frac{k_{SO_3}[SO_3]}{k_d + k_{SO_3}[SO_3] + k_{O_2}[O_2] + k_{gs}[RB]}$$

where k_d, k_{O_2}, and k_{gs} are the rate constants for $^3RB^*$ decay (6.7×10^3 s^{-1}) and quenching of $^3RB^*$ by oxygen (1.6×10^9 M^{-1} s^{-1}) and by ground state RB (7.6×10^8 M^{-1} s^{-1}), respectively, as determined by Lee and Rodgers.[9] For the conditions used by Srinivasan *et al.*[8] and using k_{SO_3} determined in this work, the fraction of $^3RB^*$ quenched by sulfite is 0.18.[20] Since both products of reaction 5 can initiate the reaction sequence 2−4, this reaction will result in significant O_2 consumption. Thus, it is not possible to quantitate the efficiency of reaction 1 by this method.

Further support for the production of $O_2^{\bullet-}$ *via* reaction 1 came from the work of Lee and Rodgers,[9] who used benzoquinone (BQ) to monitor $O_2^{\bullet-}$. BQ reacts with $O_2^{\bullet-}$ to give the semiquinone radical anion ($BQ^{\bullet-}$) with a rate constant of 9.8 × 10^8 M^{-1} s^{-1}.[21] $BQ^{\bullet-}$ absorbs maximally at 430 nm, and this absorption has been used to monitor for $O_2^{\bullet-}$.[21] To use this method to quantitate $O_2^{\bullet-}$ production, BQ must only undergo

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(16) $RB^{\bullet-}$ transfers an electron to O_2 with a rate constant of 1.1 × 10^8 M^{-1} s^{-1} in aqueous solution[17]. Electron transfer from the sulfite radical to oxygen is reaction 4.

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(19) At pH 7.8 both SO_3^{2-} and HSO_3^- are present. Thus, k_{SO_3} is an apparent rate constant with contributions from quenching by both species.

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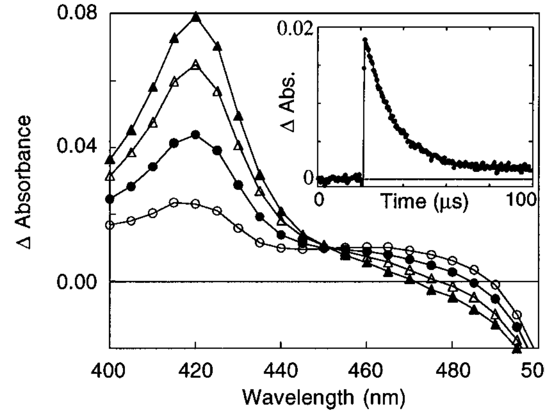

Figure 1. Time-resolved spectra of RB (5 μM) in the presence of sodium sulfite (8 mM) in phosphate buffer (0.1 M, pH 7.8), 532 nm excitation (2 mJ, 8 ns). Times after the laser pulse: 2 (○), 6 (●), 13 (△), and 36 μs (▲). (Inset) Corresponding transient decay (average of eight shots) at 620 nm of RB (5 μM) and sodium sulfite (5.8 mM).

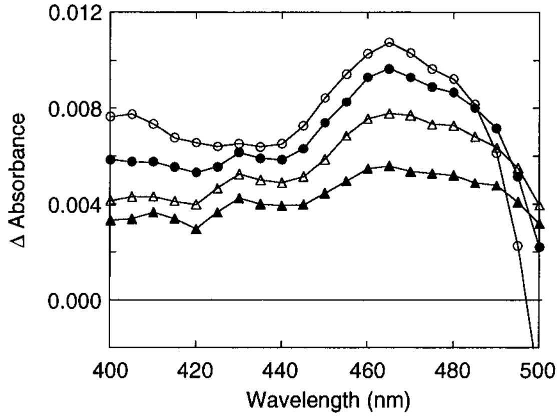

Figure 2. Time-resolved spectra of RB (5 μM) in the presence of BQ (15 μM) in phosphate buffer (0.01 M, pH 7.0), 532 nm excitation (2 mJ, 8 ns). Times after the laser pulse: 10 (○), 25 (●), 120 (△), and 355 μs (▲).

electron transfer from $O_2^{\bullet -}$, and the O_2 quenching of $^3RB^*$ must compete effectively with interactions between BQ and $^3RB^*$.

Measurement of the rate constant for $^3RB^*$ decay as a function of [BQ] using laser flash photolysis yields a value of 4.0 (±0.6) × 10^9 M^{-1} s^{-1} for the rate constant for quenching of $^3RB^*$ by BQ. Using this rate constant, the fraction of $^3RB^*$ quenched by BQ, under the conditions used by Lee and Rodgers (20 μM RB, O_2 saturated, maximum [BQ] = 100 μM), is calculated to be 0.20.[22] Hence, an efficient pathway exists by which $BQ^{\bullet -}$ may be generated under these conditions and which must be taken into account to estimate an upper limit for ϕ_{so}.

The time-resolved absorption spectra for N_2-saturated solutions of 5 μM RB in the presence of 15 μM BQ are shown in Figure 2. Under these conditions $^3RB^*$ is completely removed within 50 μs after the laser pulse, by reaction with BQ and self-quenching, reaction 6.

$$^3RB^* + RB \xrightarrow{k_{gs}} RB^{\bullet -} + RB^{\bullet +} \tag{6}$$

At the two longest times shown, the spectra have contributions from all three radical species, $RB^{\bullet -}$, $BQ^{\bullet -}$, and $RB^{\bullet +}$, which have absorption maxima at 420, 430, and 470 nm, respectively. From the absorption coefficients of these species [37 600 ($RB^{\bullet -}$),[17] 7300 ($BQ^{\bullet -}$),[23] and 21 100 M^{-1} cm^{-1} ($RB^{\bullet +}$)[17]], it can be calculated that quenching of $^3RB^*$ by BQ and RB ground state is less than 100% efficient and closer to 10% due to back electron transfer.

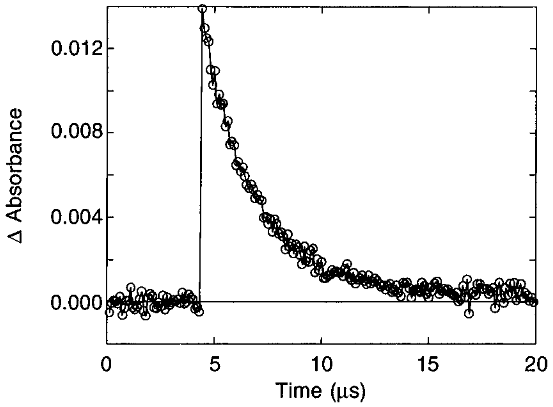

Figure 3. Transient decay (average of 16 shots) monitored at 470 nm of RB (5 μM) in phosphate buffer (0.01 M, pH 7.0), 532 nm excitation (2 mJ, 8 ns), air saturated.

Quenching of $^3RB^*$ by ground state RB, to give $RB^{\bullet -}$ and $RB^{\bullet +}$, provides a possible route to $O_2^{\bullet -}$ formation since it may be followed by reaction 7, which has a rate constant of 1.5 × 10^8 $M^{-1}s^{-1}$.[17] Thus, the apparent yield of $O_2^{\bullet -}$ is dependent on

$$RB^{\bullet -} + O_2 \xrightarrow{k_7} RB + O_2^{\bullet -} \tag{7}$$

the RB concentration. At the maximum [RB] used by Lee and Rodgers (100 μM), this pathway accounts for quenching of ≈1% of the $^3RB^*$. Without knowing the fraction of $BQ^{\bullet -}$ that is generated by electron transfer from species other than $O_2^{\bullet -}$, it is not possible to use BQ to measure ϕ_{so}.

A more direct method for determining the yield of $O_2^{\bullet -}$ produced by reaction 1 is to measure the absorption of $RB^{\bullet +}$ at 470 nm after the decay of $^3RB^*$. Laser flash photolysis of an air-saturated, RB (5 μM) solution shows a residual absorption that is 3% of the initial $^3RB^*$ absorption (Figure 3).[24] This corresponds to a $RB^{\bullet +}$ yield of 0.5%, based on a $RB^{\bullet +}$ absorption coefficient of 21 100 M^{-1} cm^{-1},[17] and represents the maximum the yield of $O_2^{\bullet -}$ since $RB^{\cdot +}$ can also be formed by reaction 6. In organic solvents such as methanol where the oxygen concentration is 10-fold higher than in water, no residual absorption is observed at 470 nm. The residual absorption was also removed when the RB was solubilized in 2% aqueous Triton X 100 detergent micelles, which effectively prevents ground state quenching.

These results strongly suggest that in aqueous solution or a biological environment where RB is bound to a membrane or macromolecule, the formation of $O_2^{\bullet -}$ is a very inefficient process. Electron transfer to O_2 from a reduced substrate (produced by electron transfer from $^3RB^*$) could generate $O_2^{\bullet -}$ but requires an O_2 concentration low enough not to quench $^3RB^*$ by energy transfer but high enough to intercept the reduced substrate.

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