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Quantification of Singlet Oxygen Production in the Reaction of Superoxide with Hydrogen Peroxide Using a Selective Chemiluminescent Probe

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Superoxide radical anion ($O_2^{-\bullet}$)[1] and hydrogen peroxide (H_2O_2)[2] are formed intracellularly, and the reaction of these two reactive oxygen species (ROS) has been studied for decades in an effort to explain their observed "toxic synergism".[3,4] Both singlet oxygen ($^1O_2$) and hydroxyl radical ($^\bullet$OH) have been proposed as highly cytotoxic products of this reaction. Singlet oxygen is known to oxidize a variety of biological substrates, such as proteins,[5] certain amino acids,[6] and nucleic acids.[7] Hydroxyl radical is a nonspecific oxidant, reacting with proteins and free amino acids with rate constants ranging from 10^7 to 10^{10} M^{-1} s^{-1}.[8]

The formation of both $^1O_2$ and $^\bullet$OH in the reaction of $O_2^{-\bullet}$ with H_2O_2 has been proposed to occur via the so-called Haber−Weiss or Haber−Willstätter reaction[9] (eq 1), in which a fraction of the O_2 is produced as $^1O_2$. The biological relevance of this reaction has been debated for at least four decades.[4,10−12]

$$H_2O_2 + O_2^{-\bullet} \rightarrow O_2 + {}^\bullet OH + {}^-OH \qquad (1)$$

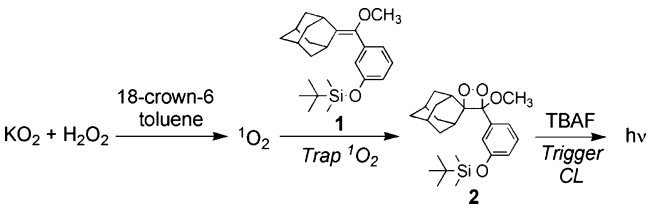
In 1975, Kellogg and Fridovich suggested that $^1O_2$ could be formed in the Haber−Weiss reaction and provided experimental evidence of its production in the reaction of xanthine oxidase with acetaldehyde in an aqueous system, which simultaneously generates $O_2^{-\bullet}$ and H_2O_2.[13] Experimental evidence of $^1O_2$ production in the Haber−Weiss reaction has also been found in aprotic solvents,[14] which are useful models of the nonaqueous hydrophobic environment of lipid bilayers.[15]

In this study, we used a sensitive chemiluminescent probe that selectively reacts with $^1O_2$ in the presence of $O_2^{-\bullet}$ and H_2O_2 to quantify the production of $^1O_2$ in the reaction of these two ROS. The trap-and-trigger probe, which we have described previously,[16] is based on a stable dioxetane precursor (**1**, Scheme 1). This detection method builds upon the work of Schaap,[17] Adam,[18] and others,[19] who have reported a series of spiroadamantylidene-substituted dioxetanes that are unusually stable and require a chemical trigger to initiate their chemiluminescent decomposition. In this detection scheme, $^1O_2$ is trapped in the form of a stable dioxetane (**2**, Scheme 1), which is quantified by its chemiluminescence (CL) signal, triggered by the addition of tetra-*n*-butylammonium fluoride (TBAF).

The reaction of $O_2^{-\bullet}$ with H_2O_2 was carried out in aprotic solvent (toluene) to take advantage of the greater stability and higher reactivity of $O_2^{-\bullet}$ under such conditions.[20] To circumvent the complications associated with heterogeneous reaction conditions or the presence of water, a homogeneous solution of H_2O_2 (0.032 M) was prepared in toluene by the oxidation of 2-ethylanthrahydroquinone (2-EAHQ) and purified by distillation (see Supporting Information).

The formation of dioxetane **2** in the reaction of KO_2 (2 mM, solubilized with 18-crown-6 ether; see Supporting Information for the determination of the dissolved $O_2^{-\bullet}$ concentration) with H_2O_2 (10 mM) in toluene at 25 °C in the presence of 100 μM probe **1** was followed by analyzing aliquots of the reaction mixture over a period of 90 min (Figure 1, circles). The formation of dioxetane **2** followed apparent first-order kinetics with an observed rate constant, k_{obs}, of $(2.1 \pm 0.3) \times 10^{-3}$ s^{-1}. The yield of $^1O_2$ under these conditions was calculated to be $(4.0 \pm 0.4) \times 10^{-6}$ M, which corresponds to a yield of (0.20 ± 0.03)% relative to the initial $O_2^{-\bullet}$ concentration.

Scheme 1



The formation of $^1O_2$ in this system was supported by experiments in which its lifetime was increased through the use of deuterated solvent and decreased by the addition of a $^1O_2$ quencher. The possible reaction and deactivation pathways of $^1O_2$ in this system are illustrated in Scheme 2. When the reaction of $O_2^{-\bullet}$ with H_2O_2 was carried out in 57% toluene-d_8 (Figure 1, squares), the observed enhancement in the $^1O_2$ yield (1.7) closely matched the enhancement predicted from the$^1O_2$ lifetimes in the two solvent mixtures (2.0). The formation of $^1O_2$ was also predictably inhibited by the addition of 1,4-diazabicyclo[2.2.2]octane (DABCO), a known $^1O_2$ quencher[21] (Figure 1, triangles).

It has been proposed that the Haber−Weiss reaction (eq 1) does not occur in the absence of a metal catalyst.[4,11] We found no evidence for the participation of trace metal impurities in the formation of $^1O_2$ from KO_2 and H_2O_2, as there were no differences in the apparent kinetics or $^1O_2$ yield in the presence and in the absence of diethylenetriaminepentaacetic acid (DTPA), a metal chelator. However, in the presence of a relatively high amount of *added* Fe(II) acetate (100 μM), a 6-fold increase in the $^1O_2$ yield and an almost 2-fold increase in the apparent first-order rate constant were observed (see Supporting Information), suggesting that the reaction of $O_2^{-\bullet}$ with H_2O_2 to produce $^1O_2$ can be catalyzed by Fe(II) and possibly other redox-active metals. It is unclear from these experiments how the Fe(II) facilitates the production of $^1O_2$, although it has been suggested that it can catalyze the reaction by acting as a redox mediator.[4,9]

The potential formation of $^\bullet$OH in the reaction of $O_2^{-\bullet}$ with H_2O_2 under the conditions used in this study was also investigated. Hydroxyl radical is known to react with toluene with a rate constant of 8.1×10^9 M^{-1} s^{-1} to preferentially form *o*-, *m*-, and *p*-cresols (as opposed to benzyl radical);[23] the relative yields of these products are reported to be 0.84, 0.41, and 1.0, respectively.[24] A spectrophotometric detection method using Gibbs's reagent[25] (2,6-dichloroquinone-4-chloroimide) was used to quantify the total cresol concentration as an indicator of $^\bullet$OH production. By this method,

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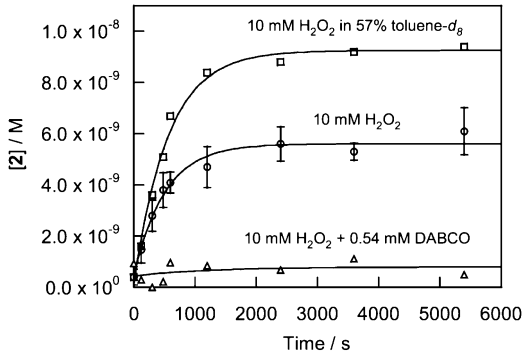
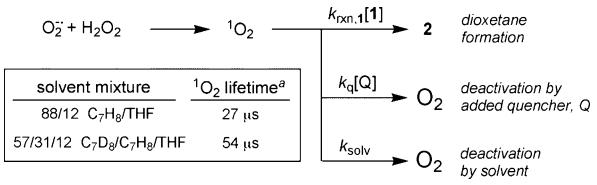


Figure 1. Production of dioxetane **2** during exposure of **1** (100 μM) to $O_2^{-\bullet}$ (2 mM) and H_2O_2 (10 mM) at 25 °C in toluene (○), in 57% toluene-d_8 (□), or in toluene with 0.54 mM DABCO (△). Data are fit to a monoexponential growth function. Error bars represent one standard deviation from four replicate measurements.

Scheme 2



solvent mixture	$^1O_2$ lifetime[a]
88/12 C_7H_8/THF	27 μs
57/31/12 C_7D_8/C_7H_8/THF	54 μs

[a] Lifetimes for $^1O_2$ were calculated from the fractional solvent composition and published lifetimes in pure solvents.[22]

the total cresol concentration after the reaction of $O_2^{-\bullet}$ with H_2O_2 in toluene was found to be less than the detection limit, 200 nM (see Supporting Information). With an upper limit of 200 nM, the yield of $^\bullet$OH can be no more than 0.01% relative to the initial $O_2^{-\bullet}$ concentration. This result suggests that the Haber−Weiss mechanism is not important in the reaction of $O_2^{-\bullet}$ with H_2O_2 under these conditions.

Another mechanism has been proposed for the reaction of $O_2^{-\bullet}$ with H_2O_2 in aprotic solvent in which H_2O_2 acts as a proton donor for $O_2^{-\bullet}$ (eqs 2 and 3).[26]

$$H_2O_2 + O_2^{-\bullet} \rightarrow HO_2^- + HO_2^\bullet \qquad (2)$$

$$HO_2^\bullet + O_2^{-\bullet} \rightarrow HO_2^- + O_2 \qquad (\text{or } {}^1O_2) \qquad (3)$$

If this mechanism is the source of $^1O_2$ in this study, one would expect any acid with a pK_a similar to that of H_2O_2 (10.7 in *N*,*N*-dimethylformamide (DMF)[27]) to also react with $O_2^{-\bullet}$ to produce $^1O_2$. However, when H_2O_2 was replaced with 2-nitrobenzoic acid, which has a pK_a of 9.9 in DMF,[28] no $^1O_2$ production was observed. Additionally, no $^1O_2$ was observed in the reaction of $O_2^{-\bullet}$ with another soluble proton donor, *tert*-butyl alcohol (see Supporting Information). Thus, a simple acid−base reaction between $O_2^{-\bullet}$ and H_2O_2 does not appear to be sufficient to describe the mechanism of $^1O_2$ production in this system.

Estimated biological concentrations of H_2O_2 and $O_2^{-\bullet}$ have been reported as $\leq 10^{-5}$ M and ca. 10^{-9} M (pH 7, aqueous), respectively.[29] As H_2O_2 is produced in the disproportionation of $O_2^{-\bullet}$, these species can also be expected to be co-localized in biological systems. Our results indicate that these species react to produce $^1O_2$ but with very low efficiency, and we found no evidence for the production of $^\bullet$OH. The low yields of $^1O_2$ and $^\bullet$OH suggest that their formation in the uncatalyzed reaction of $O_2^{-\bullet}$ with H_2O_2 should be relatively unimportant in biological systems, even in water-free hydrophobic environments where the stabilities and reactivities of these ROS may be greater than in aqueous environments.

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Supporting Information Available: Experimental details for the preparation of 2-EAHQ and the generation of H_2O_2 in toluene; the reactions of $O_2^{-\bullet}$ with H_2O_2, 2-nitrobenzoic acid, and *tert*-butyl alcohol; and the detection of cresols using Gibbs's method. This material is available free of charge via the Internet at http://pubs.acs.org.

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