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Singlet molecular oxygen production in the reaction of peroxynitrite with hydrogen peroxide

Paolo Di Mascio[a,*], Etelvino J.H. Bechara[a], Marisa H.G. Medeiros[a], Karlis Briviba[b], Helmut Sies[b]

[a]*Instituto de Química, Departamento de Bioquímica, Universidade de São Paulo, CP 20780, CEP 01498-970, São Paulo, Brazil*
[b]*Institut für Physiologische Chemie I, Heinrich-Heine-Universität Düsseldorf, PO Box 101007, D-40001 Düsseldorf, Germany*

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Abstract Peroxynitrite and hydrogen peroxide are mediators of cytotoxicity. This study shows that the peroxynitrite anion reacts with hydrogen peroxide to release oxygen accompanied by emission of chemiluminescence (CL). Direct characterization of this light emission attributes it to the transition of singlet molecular oxygen to the triplet ground state. Chemiluminescence was monitored: (i) by dimol light emission in the red spectral region (>610 nm) using a red-sensitive photomultiplier; and (ii) by monomol light emission in the infrared (1270 nm) with a liquid nitrogen-cooled germanium diode. These properties of photoemission and the enhancing effect of deuterium oxide on CL intensity as well as the quenching effect of sodium azide are diagnostic of molecular oxygen in the excited singlet state. For comparison, singlet molecular oxygen arising from the thermolysis of the water-soluble endoperoxide of 3,3'-(1,4-naphthylidene)dipropionate or from the hypochlorite/H_2O_2 system was also monitored. These novel observations identify a potential singlet oxygen-dependent mechanism contributing to cytotoxicity mediated by peroxynitrite and hydrogen peroxide.

Key words: Singlet molecular oxygen; Peroxynitrite; Nitric oxide; Hydrogen peroxide; Chemiluminescence; Near-infrared emission

1. Introduction

Peroxynitrite ($ONOO^-$) is a biologically active species produced by the reaction of the superoxide anion radical ($O_2^{-\bullet}$) with nitric oxide ($NO^\bullet$) [1–3]. Nitric oxide, identified as the endothelium-derived relaxing factor [4], is formed by conversion of L-arginine to L-citrulline by an $NO^\bullet$ synthase. Endothelial cells, macrophages, neutrophils and neuronal cells have been shown to produce $NO^\bullet$ and $ONOO^-$ [5]. It has been suggested that decomposition of $ONOO^-$ generates the hydroxyl radical, a strong oxidant [6,7]. Recently, Noronha-Dutra et al. [8] reported that $NO^\bullet$ reacts with H_2O_2 accompanied by light emission in the visible spectral region with the features of singlet dioxygen, O_2 ($^1\Delta_g$), an excited state of molecular oxygen.

We studied here the oxygen-generating reaction of $ONOO^-$ with H_2O_2, using chemiluminescence (CL) measurement of the dimol (reaction 1) and monomol (reaction 2) light emission in the visible and infrared spectral regions, respectively, to provide evidence for the formation of O_2 ($^1\Delta_g$) in this system.

$$O_2\ (^1\Delta_g) + O_2\ (^1\Delta_g) \rightarrow 2\ O_2\ (^3\Sigma_g^-) + h\nu\ (634 \text{ and } 703 \text{ nm}) \quad (1)$$

$$O_2\ (^1\Delta_g) \rightarrow O_2\ (^3\Sigma_g^-) + h\nu\ (1270 \text{ nm}) \quad (2)$$

We suggest that, in analogy to the reaction of peroxybenzoate with H_2O_2 [9], $ONOO^-$ reacts with H_2O_2 to produce dioxygen, and that the oxygen released is present, at least in part, in the excited singlet state.

2. Materials and methods

Peroxynitrite was synthesized in a quenched flow reactor assembled according to Radi et al. [10]. In brief, separate solutions of 0.6 M $NaNO_2$ and 0.6 M HCl plus 0.7 M H_2O_2 were pumped with a peristaltic pump at 13 ml/min into a 4 mm Y-shaped glass tube. The acid-catalyzed reaction of nitrous acid with H_2O_2 forms peroxynitrous acid, which was deprotonated by pumping 3 M NaOH at the same flow rate into a T junction positioned at 2.5 cm downstream. Excess H_2O_2 was removed by passage over granular MnO_2. The solution was then filtered twice and frozen at −20°C for 5 days. Peroxynitrite forms a dark yellow top layer due to freeze fractionation, which was stored for further experiments. This solution contained 100–150 mM $ONOO^-$ as determined by absorbance at 302 nm (ε_{302} = 1,670 $M^{-1} \cdot cm^{-1}$) [11].

Low-level CL was measured with a single-photon counting system, described elsewhere [12], equipped with a red-sensitive photomultiplier, cooled to −25°C by a thermoelectric cooler. Selective light emission at wavelengths >610 nm was obtained by a cut-off filter placed between the cuvette and the photomultiplier tube. Infrared emission of O_2 ($^1\Delta_g$) was measured with a liquid nitrogen-cooled germanium photodiode detector, sensitive in the spectral region from 800 nm to 1800 nm with a detector area of 0.25 cm^2 and a sapphire window, as described elsewhere [13].

All sample solutions were in a thermostated glass cuvette of (35 mm × 6 mm × 55 mm), CL was monitored after the injection (time = 0) of a solution of $ONOO^-$ (Fig. 1) to H_2O_2 in 0.5 M sodium phosphate buffer, pH 7.4, the final pH was 8. In the experiments shown in Fig. 2, peroxynitrite and H_2O_2 were present and the reaction started by the addition of acetic acid (time = 0), the final pH was 6. The intensity of the CL was pH-dependent, with a maximum Ge-diode signal at pH 8–9. When the reaction was performed at pH 1–2 or 13–14 no signal was observed. For comparison, O_2 ($^1\Delta_g$) generated chemically was observed upon thermal decomposition of the endoperoxide of the water-soluble disodium salt of 3,3'-(1,4-naphthylidene)dipropionate ($NDPO_2$) into the parent compound and molecular oxygen or in the hypochlorite/H_2O_2 system. The decomposition of $NDPO_2$ is temperature-dependent and slow. In contrast, the O_2 ($^1\Delta_g$) yield in the hypochlorite/H_2O_2 system is almost quantitative but very fast compared with the thermodecomposition of $NDPO_2$ [14].

Saturated $NO^\bullet$ solutions in H_2O were prepared by bubbling for 2 h with N_2, then 30 min with helium and 10 min with $NO^\bullet$ gas. The solutions were kept on ice and used immediately. Solutions of $NO^\bullet$ or SIN-1 (16 mM final concentration in ethanol) were injected directly into the sample containing 15 mM H_2O_2 while CL was being measured. Oxygen evolution was measured with a Clark-type electrode at room temperature.

Nitric oxide was purchased from Linde (Düsseldorf, Germany), SIN-1 was a kind gift of Cassella-Riedel Co. (Frankfurt, Germany), other chemicals were obtained from Sigma (Deisenhofen, Germany). The experiments were repeated several times.

*Corresponding author. Fax: (55) (11) 815-5579.
E-mail: pdmascio@quim.iq.usp.br

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3. Results and discussion

The mixture of $ONOO^-$ and H_2O_2 produces a fast increase in light emission at wavelengths identified for the chemiluminescence of O_2 ($^1\Delta_g$) (see reactions 1 and 2). As shown in Fig. 1A, trace 3, $ONOO^-$ injected into a nitrogen-purged solution of H_2O_2 in phosphate buffer produced CL in the red region (>610 nm). For comparison, O_2 ($^1\Delta_g$) was also generated by the reaction of hypochlorite/H_2O_2 (Fig. 1A, trace 2) and by the thermodissociation of $NDPO_2$ (Fig. 1A, trace 1).

The monomol light emission in the peroxynitrite/H_2O_2 reaction at 1270 nm is shown in Fig. 1B, trace 3. Likewise, the O_2 ($^1\Delta_g$) production from hypochlorite/H_2O_2 and from $NDPO_2$ are shown in traces 2 and 1, respectively. A calibration of the rate of O_2 ($^1\Delta_g$) production is performed on the basis of the rate of generation of NDP: in the experiment of trace 1 in Fig. 1B,

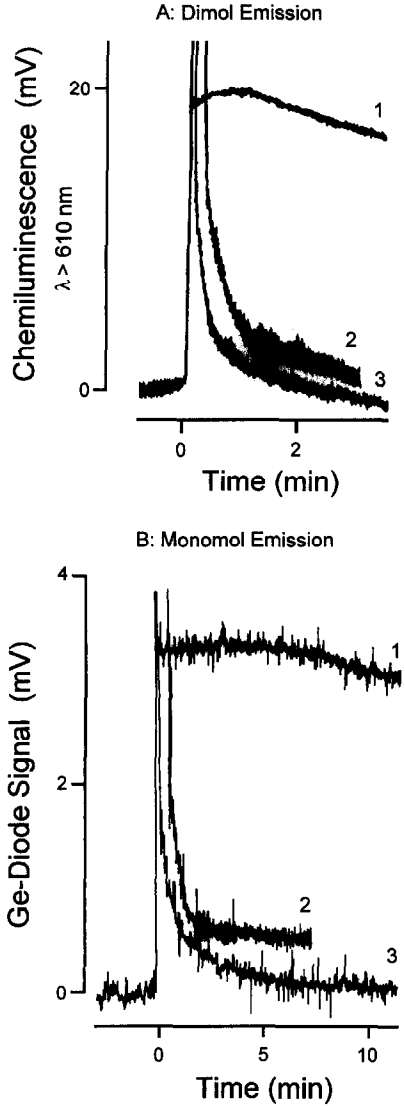


Fig. 1. Dimol (A) and monomol (B) light emission of O_2 ($^1\Delta_g$) generated by: (1) the thermodissociation of $NDPO_2$, (2) the hypochlorite/H_2O_2, and (3) the peroxynitrite/H_2O_2 systems. (1) $NDPO_2$ was 5 mM, (2) 10 mM hypochlorite was injected to 20 mM H_2O_2 and (3) $ONOO^-$ (5 mM) was injected into a 4 ml of 20 mM H_2O_2. Conditions: 0.5 M sodium phosphate buffer (final pH, 8), temperature was 37°C.

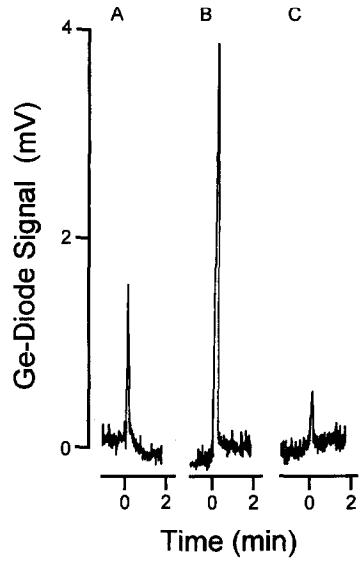


Fig. 2. Effect of D_2O and NaN_3 on the monomol emission generated by peroxynitrite/H_2O_2. Time course of the Ge-diode signal in: (A) H_2O, (B) 70% D_2O, and (C) 10 mM NaN_3 in 70% D_2O. A solution of acetic acid was injected in a 4.5 ml solution of 1.4 mM $ONOO^-$/14 mM H_2O_2 (final pH, 6), temperature was 37°C.

thermodissociation of $NDPO_2$ yielded 25 μM O_2 ($^1\Delta_g$) $min^{-1} \cdot mV^{-1}$ [14], so that we roughly estimate a flash of O_2 ($^1\Delta_g$) production of 50 μM O_2 ($^1\Delta_g$) for the reaction peroxynitrite/H_2O_2 shown in trace 3 in Fig. 1B. The generation of O_2 ($^1\Delta_g$) reaction of hypochlorite/H_2O_2 is also shown (Fig. 1B, trace 2). Neither $ONOO^-$ nor H_2O_2 nor hypochlorite elicited CL when present alone. The intensity of the CL was increasing with the $ONOO^-$ concentration tested up to 20 mM (not shown).

For further characterization, the effects of deuterated water and of azide were examined. In these experiments, the reaction was initiated by the addition of acetic acid. Upon replacement of H_2O by 70% D_2O, the CL intensity increased 8-fold in the dimol emission and 5-fold in the monomol emission at 1270 nm (Fig. 2B). This is consistent with the fact that the lifetime of O_2 ($^1\Delta_g$) increases in D_2O by about this factor [15]. As expected for O_2 ($^1\Delta_g$) as the emitter in the peroxynitrite/H_2O_2 reaction, sodium azide effectively quenched the signal in Fig. 2C compare with Fig. 2B.

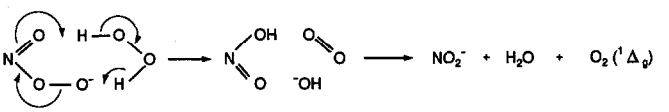
To assess whether the CL (dimol and monomol emission) was dependent on hydroxyl radical formation, the scavengers dimethyl sulfoxide (10% v:v) and mannitol (100 mM) were used: in both cases, CL was not affected. Addition of a metal chelator, diethylenetriamine pentaacetic acid (DETAPAC, 20 mM), also did not affect the germanium diode signal.

The evolution of oxygen was followed in further experiments in 0.1 M phosphate buffer solutions, pH 7.4, previously purged with nitrogen, which contained 1 mM H_2O_2 and 0.5 mM $ONOO^-$ at room temperature (data not shown). The release of oxygen in the reaction was previously demonstrated by Mahoney [16].

Using the two CL methods described here, we also investigated the reaction of $NO^\bullet$ with H_2O_2 to produce O_2 ($^1\Delta_g$) as described by Noronha-Dutra et al. [8]. When deoxygenated solutions of $NO^\bullet$ or SIN-1 as a source of $NO^\bullet$ plus $O_2^{-\bullet}$ [17] were

injected into a solution of H_2O_2, no CL was recorded within the limits of detection.

Taken together, these observations serve as qualitative evidence for O_2 ($^1\Delta_g$) production by the reaction of $ONOO^-$ with H_2O_2. Further work is required to determine the yield of O_2 ($^1\Delta_g$) production in this reaction. Inspired by common chemical features shared by the peroxybenzoate/H_2O_2 system [9] and the reaction studied here, we envisage a concerted mechanism for O_2 ($^1\Delta_g$) formation via the *cis*-configuration of $ONOO^-$. In fact, the *cis* form of $ONOO^-$ was recently described to predominate at the more alkaline pH [18].



(Scheme I).

This reaction may be involved in the cytotoxicity of macrophages [19] and provides further insight into the cytotoxic potential of O_2 ($^1\Delta_g$).

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