SINGLET OXYGEN PRODUCTION BY LACTOPEROXIDASE: HALIDE DEPENDENCE AND QUANTITATION OF YIELD†

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Summary

Chemiluminescence at 1268 nm was used to measure singlet oxygen production in the lactoperoxidase–(hydrogen peroxide)–halide systems. The use of a liquid-nitrogen-cooled germanium detector permitted the measurement of singlet oxygen production rates as low as 30 nmol min^{-1}. Under optimal conditions using bromide ion as a substrate, lactoperoxidase produced singlet oxygen in stoichiometric amounts consistent with the mechanism

$$H_2O_2 + H^+ + Br^- \xrightarrow{\text{lactoperoxidase}} H_2O + HOBr$$

$$H_2O_2 + HOBr \longrightarrow H_2O + H^+ + Br^- + O_2(^1\Delta_g)$$

The oxidized bromine species, tribromide ion, bromine and hypobromous acid, were not significant final reaction products.

No 1268 nm chemiluminescence was detected with chloride ion or iodide ion. Lactoperoxidase does not oxidize chloride ion. The absence of 1268 nm chemiluminescence does not rule out some singlet oxygen production in the lactoperoxidase–(hydrogen peroxide)–iodide system, since iodine is known to quench singlet oxygen.

1. Introduction

In a preliminary report from this laboratory, singlet oxygen production was demonstrated in the lactoperoxidase (LPO)–(hydrogen peroxide)–bromide system using 1268 nm chemiluminescence [1]. This observation was confirmed by Khan [2]. Further details about the LPO–(hydrogen peroxide)–halide systems and the experimental method are now reported.

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2. Experimental details

2.1. Chemiluminescence spectrometer

The chemiluminescence spectrometer has been described previously [1]. An EO-817L liquid-nitrogen-cooled germanium detector (North Coast Optical Systems and Sensors, Santa Rosa, CA) was used to measure the 1268 nm chemiluminescence. For studies of the enzymatic systems, the integral of the chemiluminescence intensity over the total reaction period is reported. This is expressed as a percentage of a reference chemical source of singlet oxygen, the hydrogen peroxide plus hypobromous acid reaction. "Dimol" chemiluminescence was detected with an RCA 8852 photomultiplier cooled to −20 °C (RCA, Lancaster, PA). A number 29 Kodak Wratten filter (Eastman Kodak Company, Rochester, NY) was placed in front of the photomultiplier to block a small amount of shorter wavelength light not due to singlet oxygen.

2.2. Flow system studies

A flow system using the hydrogen peroxide plus hypochlorous acid reaction was used to provide a continuous source of singlet oxygen at known production rates. A model 600-910/920 syringe pump (Harvard Apparatus Company, Natick, MA) pushed the reactants through two 17-gauge Teflon tubes. The tubes were adjacent and parallel; their openings were cut at a 45° angle inward to promote mixing. The two tubes were placed at the center of a 12 mm Pyrex tube which served as an outflow channel.

2.3. Oxidized bromine species

Tribromide ion concentrations were measured using 267 nm absorbance [3, 4]. The total concentration of the oxidized bromine species, tribromide ion, bromine and hypobromous acid, was calculated from an experimentally determined value of 4.3×10^4 M^{-1} cm^{-1} for the extinction coefficient of tribromide ion, an association constant of 14.4 M^{-1} for tribromide ion and a hydrolysis constant of 7.2×10^{-9} M^{-2} for bromine [3, 5, 6].

2.4. Reagents

LPO ($A_{412\,nm}/A_{280\,nm}$ = 0.81; specific activity, 66 units mg^{-1} using pyrogallol as a substrate with one unit forming 1 mg purpurogallin in 20 s at pH 6 at 20 °C), bovine serum albumin (BSA) and histidine were obtained from Sigma, St. Louis, MO. LPO was assayed at 412 nm using an absorption coefficient of 1.14×10^5 M^{-1} cm^{-1} [7]. Hypochlorous acid was distilled under reduced pressure from a 5.25% commercial solution (Clorox) acidified to pH 8 with phosphoric acid [8]. It was stored at 4 °C, for periods less than 1 week, as a 300 mM solution with the pH increased to 11 by the addition of sodium hydroxide. On the day of use, it was assayed at 292 nm using an absorption coefficient of 391 M^{-1} cm^{-1} [9]. Solutions of oxidized bromine species were prepared by the addition of hypochlorous acid to buffers containing excess sodium bromide [5]. These solutions were used

immediately. Hydrogen peroxide was diluted from a 30% stabilized reagent grade solution (Superoxol, J. T. Baker, Phillipsburgh, NJ). Stock solutions were assayed iodometrically [10]. All other inorganic chemicals were reagent grade. Water was glass distilled.

2.5. *Reaction conditions*

All experiments were done at 25 °C. For flow system studies, sodium hypochlorite was diluted in pH 9, 100 mM sodium-carbonate buffer. Because of the instability of dilute sodium hypochlorite, these solutions were used immediately [11]. Hydrogen peroxide was diluted in distilled water, since it decomposes in basic solution [12].

Studies of LPO were done in buffered solutions (100 mM sodium phosphate of pH 3, 6 and 7; 100 mM sodium acetate of pH 4, 4.5 and 5) containing the desired concentration of sodium halide. A dark adapted test-tube containing LPO in 1.5 ml of buffer was placed in the spectrometer. The reaction was initiated by the rapid injection of an additional 1.5 ml of buffer containing hydrogen peroxide. For measurements of chemiluminescence in the hydrogen peroxide plus hypobromous acid reaction, hypobromous acid in 1.5 ml of buffer was placed in the spectrometer. The reaction was initiated by the rapid injection of 1.5 ml of buffer containing hydrogen peroxide.

2.6. *Error analysis*

All experiments were done in triplicate. Data are reported as the mean plus or minus the standard error.

3. Results

3.1. *Spectrometer sensitivity*

Flow system studies using the hydrogen peroxide plus hypochlorous acid reaction were used to measure the sensitivity of the spectrometer for singlet oxygen and to compare the relative sensitivity of the dimol and monomol bands. At pH 9, this reaction is fast and the yield of singlet oxygen is 100% [8]. The flow rates were set so that the reaction was complete before the reactants left the field of view of the light detectors. Figure 1 shows that, as expected, the dimol chemiluminescence varied as the square of the singlet oxygen production rate (slope of least-squares line, 1.97 for a logarithmic plot) while the monomol chemiluminescence varied directly with the singlet oxygen production rate (slope of least-squares line, 1.00) [13]. At a signal-to-noise ratio of 3:1 (1 min observation periods), the IR detector could detect singlet oxygen production rates 3% of those detected with the photomultiplier. The spectrometer could measure singlet oxygen production rates of 30 nmol min^{-1}. In systems where deuterium oxide can be used as a solvent, a thirtyfold enhancement of the chemiluminescence occurs and the detection limit drops to 1 nmol min^{-1} [1].

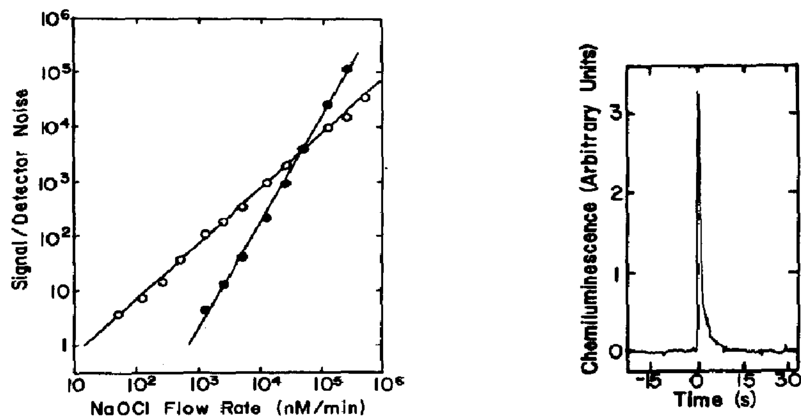

Fig. 1. Relative sensitivity of dimol and monomol bands to singlet oxygen (flow rates, 3.8 ml min^{-1} 300 mM hydrogen peroxide in unbuffered water, 3.8 ml min^{-1} sodium hypochlorite in 100 mM sodium carbonate (pH 9.5)): ●, dimol bands measured with an RCA 8852 photomultiplier; ○, monomol band at 1268 nm measured with an EO-817L detector. The detector noise was an r.m.s. value for 1 min observation periods for both detectors.

Fig. 2. Typical 1268 nm chemiluminescence signal (100 mM sodium acetate; pH 5; 225 nM LPO; 10 mM sodium bromide; 1 mM hydrogen peroxide).

3.2. Lactoperoxidase–(hydrogen peroxide)–halide systems

Chemiluminescence at 1268 nm was detected with bromide ion as a substrate but not with chloride ion or iodide ion. The chemiluminescence was less than 2% of that predicted (see Section 4) for the following 25 sets of conditions: pH 3, 4, 5, 6 and 7; 100 mM sodium chloride; 0.03, 0.1, 1.0 and 10 mM sodium iodide; 2 mM hydrogen peroxide; 900 nM LPO. Figure 2 shows a typical chemiluminescence signal for the LPO–(hydrogen peroxide)–bromide system. Figure 3 demonstrates the pH dependence of the chemiluminescence. High bromide concentrations shifted the optimal pH to higher values. As illustrated in Fig. 4, the chemiluminescence was reduced at both high and low bromide ion concentrations. Table 1 shows the variation in chemiluminescence with LPO concentration. The decrease in chemiluminescence at low enzyme concentrations was due to inactivation of the enzyme, since the injection of additional enzyme resulted in a second light peak. The drop-off at high concentrations was probably due to quenching of singlet oxygen by LPO or the consumption of hypobromous acid by reaction with LPO, since the addition of comparable amounts of BSA also reduced the chemiluminescence. Histidine, a known singlet oxygen quencher, also inhibited the chemiluminescence. As shown in Fig. 5, the singlet oxygen production was proportional to the hydrogen peroxide concentration, but the efficiency of singlet oxygen generation dropped off somewhat at the highest concentrations studied. At still higher concentrations the process is much less efficient [1].

Oxidized bromine species were not major final reaction products. Under conditions of efficient singlet oxygen production (pH 5, 100 mM

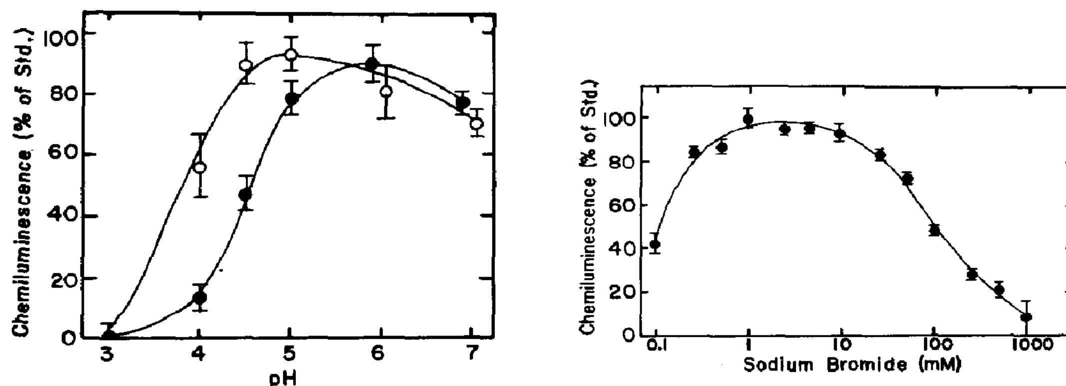

Fig. 3. Effect of pH on 1268 nm chemiluminescence for the LPO–(hydrogen peroxide)–bromide system (1 mM hydrogen peroxide; 900 nM LPO; 100 mM sodium phosphate (pH 3, 6 and 7); 100 mM sodium acetate (pH 4, 4.5 and 5): ○, 10 mM sodium bromide; ●, 100 mM sodium bromide. The chemiluminescence is expressed as a percentage of a chemical standard, 0.5 mM hydrogen peroxide plus 0.5 mM hypobromous acid, having the same pH as each LPO data point.

Fig. 4. Effect of bromide ion concentration on 1268 nm chemiluminescence in the LPO–(hydrogen peroxide)–bromide system (pH 4.5; 100 mM sodium acetate; 1 mM hydrogen peroxide; 900 nM LPO). The chemiluminescence is expressed as a percentage of the chemical standard, hydrogen peroxide plus hypobromous acid (0.5 mM each; 9 mM sodium bromide; 100 mM sodium acetate; pH 4.5).

TABLE 1

Effect of enzyme concentration on 1268 nm chemiluminescence in the lactoperoxidase–(hydrogen peroxide)–bromide system[a]

Enzyme concentration (nM)	*Chemiluminescence*	
	Integral[b] (%)	*Peak intensity* (arbitrary units)
22	25 ± 4	2.4 ± 0.4
45	65 ± 6	6 ± 1
90	92 ± 6	13 ± 1
220	100 ± 6	31 ± 2
450	98 ± 5	48 ± 1
900	91 ± 6	57 ± 1
1800	85 ± 6	67 ± 5
3600	69 ± 5	59 ± 2
220[c]	42 ± 5	12 ± 1
220[d]	7 ± 3	2.5 ± 0.5

[a]Conditions: pH 5; 100 mM sodium acetate; 10 mM sodium bromide; 1 mM hydrogen peroxide.
[b]The integral of the chemiluminescence intensity is expressed as a percentage of a chemical standard of 0.5 mM hydrogen peroxide, 0.5 mM hypobromous acid, pH 5, 100 mM sodium acetate and 10 mM sodium bromide.
[c]0.4 mg ml^{-1} BSA added.
[d]2 mM histidine added.

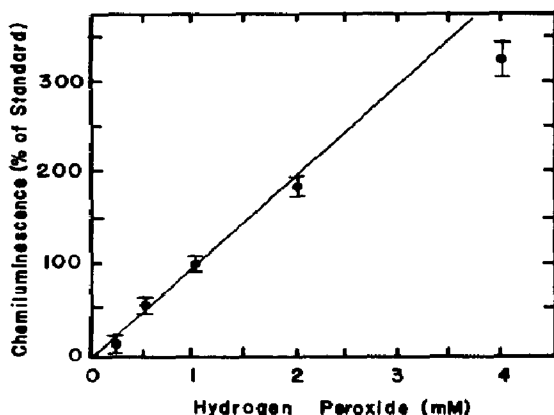

Fig. 5. Effect of hydrogen peroxide concentration on 1268 nm chemiluminescence in the LPO–(hydrogen peroxide)–bromide system (pH 5; 100 mM sodium acetate; 450 nM LPO; 20 mM sodium bromide). The chemiluminescence is expressed as a percentage of a chemical standard, 0.5 mM hydrogen peroxide plus 0.5 mM hypobromous acid (pH 5; 100 mM sodium acetate; 20 mM sodium bromide): ——, chemiluminescence predicted by the Allen mechanism (see text).

sodium bromide, 450 nM LPO and 1 mM hydrogen peroxide), the concentration of oxidized bromine species was less than 3 μM at a reaction time of 1 min. In the chloroperoxidase–(hydrogen peroxide)–bromide system, low pH, high bromide ion concentrations, high enzyme concentrations and low hydrogen peroxide concentrations favored the formation of oxidized bromine species, but in an experiment run at pH 4, 300 mM sodium bromide, 900 nM LPO and 0.25 mM hydrogen peroxide, the concentration of oxidized bromine species was less than 3 μM at a reaction time of 1 min [6]. These measurements do not rule out the presence of significant concentrations of oxidized bromine species at shorter reaction times, however. The tribromide ion was highly unstable in the presence of LPO with a rapid decay in the 267 nm absorbance (data not shown). Also, absorbance measurements at short reaction times were complicated by the presence of fine bubbles which scattered the light. At a reaction time of 1 min, almost all the bubbles had dissipated and consequently made only a minimal contribution to the total absorbance.

4. Discussion

4.1. Detection of singlet oxygen in biological systems

This study confirms the suggestion of Khan and Kasha [14] that 1268 nm chemiluminescence would be a sensitive and specific test for singlet oxygen production in biological systems. With the current instrumentation, 1268 nm emission provided a thirtyfold improvement in sensitivity for singlet oxygen compared with the dimol emissions at 634 and 703 nm. Of more significance was the absence of interference from other chemilumines-

cent reactions. In the LPO–(hydrogen peroxide)–bromide system, a significant portion of the light detected with a red-sensitive photomultiplier came from sources other than singlet oxygen. In contrast, all the light around 1268 nm appeared to be due to singlet oxygen [1]. Chemiluminescence at shorter wavelengths is common in biological oxidations and often has a broad spectral distribution which may include the dimol bands [15, 16]. The lack of interference at 1268 nm is not surprising. At the low concentrations of singlet oxygen expected in biological systems, the intensity of the 1268 nm band is several orders of magnitude higher than the dimol bands [17, 18]. Consequently, the intensity of any emission causing significant interference must be much larger than one which would obscure the dimol bands.

In spite of these arguments, it is desirable at present to obtain additional supporting evidence for the assignment of emission near 1268 nm to singlet oxygen. Useful data include the following: (1) the correct spectral distribution, a band near 1268 nm, not a broad continuum; (2) a large increase in light when the system under study is placed in deuterium oxide instead of water; (3) the quenching of light by singlet oxygen quenchers or traps. All these criteria have been met for the LPO–(hydrogen peroxide)–bromide system in the current and previous studies [1, 2].

4.2. Lactoperoxidase–(hydrogen peroxide)–halide systems

Allen [19] proposed that the myeloperoxidase–(hydrogen peroxide)–bromide system produced singlet oxygen by the mechanism

$$H_2O_2 + H^+ + Br^- \xrightarrow{\text{peroxidase}} H_2O + HOBr \tag{1}$$

$$H_2O_2 + HOBr \longrightarrow H_2O + H^+ + Br^- + O_2(^1\Delta_g) \tag{2}$$

In this study singlet oxygen production in the LPO–(hydrogen peroxide)–halide systems was compared with a reference chemical source of singlet oxygen, the hydrogen peroxide plus hypobromous acid reaction. The concentrations of both the hydrogen peroxide and the hypobromous acid in the chemical reference were one-half of the concentration of hydrogen peroxide in the LPO system under study, since this represented the maximum amount of singlet oxygen that would be expected from the Allen mechanism. Because of a modest variation in singlet oxygen chemiluminescence with pH and halide concentration, the chemical reference always had the same buffer and salt concentration as the LPO system under study [6, 20].

Under optimal conditions, the LPO–(hydrogen peroxide)–bromide system produced close to 100% of the light predicted by the Allen mechanism. No chemiluminescence was detected with chloride or iodide ion. LPO cannot oxidize chloride ion. With iodide ion at acid pH, singlet oxygen is not a quantitatively important reaction product, since LPO stoichiometrically converts iodide ion to iodine [21]. At neutral pH, however, LPO demonstrates an iodide-ion-catalyzed catalatic activity which Magnusson and Taurog [22] have proposed operates by a mechanism analogous to that proposed by Allen. With iodide ion, no 1268 nm emission was detected

under a variety of conditions. This does not rule out singlet oxygen production, however, because iodine is a quencher of singlet oxygen [23].

The tribromide ion has been detected as a product of the chloroperoxidase–(hydrogen peroxide)–bromide system, but was not detected in this study of the LPO–(hydrogen peroxide)–bromide system at a reaction time of 1 min [3, 4, 6]. This was in part due to the rapid destruction of any oxidized bromine species formed by their reaction with LPO. Unfortunately, the apparatus and experimental conditions used did not permit a valid assessment of tribromide ion concentrations at shorter reaction times.

Oxidized bromine species may not be significant final reaction products for kinetic reasons. In systems of consecutive reactions, such as the Allen mechanism, the intermediate product, here hypobromous acid, will accumulate only when the first reaction (reaction (1)) is fast relative to the second (reaction (2)) [24]. In contrast with the chloroperoxidase system, where the enzyme is highly active at an acid pH at which the hydrogen peroxide plus hypobromous acid reaction is slow, LPO is most active at higher pH where the hydrogen peroxide plus hypobromous acid reaction is much faster [25, 26]. Another explanation is the formation of an enzyme-bound halogenating intermediate as has been proposed for chloroperoxidase [3].

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