

Biochemical requirements for singlet oxygen production by purified human myeloperoxidase.

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Research Article

The myeloperoxidase (MPO)-hydrogen peroxide (H_2O_2)-halide systems were found to produce chemiluminescence at 1,268 nm, a characteristic emission band for singlet oxygen (1O_2). The emission was enhanced by a factor of 29 ± 5 in deuterium oxide and was inhibited by the 1O_2 quenchers, histidine and azide ion. Inactivation of MPO with heat or with cyanide ion prevented light production. The combined weight of all data strongly supported the production of 1O_2 by these enzyme systems. The amount of 1O_2 produced was sensitive to the conditions employed. Under optimal conditions at pH 5, the MPO- H_2O_2 -bromide (Br-) system produced 0.42 ± 0.03 mol 1O_2 /mol H_2O_2 consumed, close to the theoretical value of 0.5 that was predicted by the reaction stoichiometry. In contrast, the MPO- H_2O_2 -chloride (Cl-) system was much less efficient. The maximum yield of 1O_2 was 0.09 ± 0.02 mol/mol H_2O_2 consumed and required pH 4 and 5 mM H_2O_2 . At higher pH, the 1O_2 production rapidly decreased. The yield at pH 7 was 0.0004 ± 0.0002 mol/mol H_2O_2 consumed. Enzyme inactivation was a major factor limiting the yield of 1O_2 with both Cl- and Br-. While the MPO- H_2O_2 -halide systems can efficiently produce 1O_2 , the conditions required are not physiologic, which suggests that the chemiluminescence of the stimulated neutrophil does not derive from 1O_2 generated by a MPO mechanism.

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Abstract. The myeloperoxidase (MPO)-hydrogen peroxide (H_2O_2)-halide systems were found to produce chemiluminescence at 1,268 nm, a characteristic emission band for singlet oxygen (1O_2). The emission was enhanced by a factor of 29 ± 5 in deuterium oxide and was inhibited by the 1O_2 quenchers, histidine and azide ion. Inactivation of MPO with heat or with cyanide ion prevented light production. The combined weight of all data strongly supported the production of 1O_2 by these enzyme systems. The amount of 1O_2 produced was sensitive to the conditions employed. Under optimal conditions at pH 5, the MPO- H_2O_2 -bromide (Br^-) system produced 0.42 ± 0.03 mol 1O_2 /mol H_2O_2 consumed, close to the theoretical value of 0.5 that was predicted by the reaction stoichiometry. In contrast, the MPO- H_2O_2 -chloride (Cl^-) system was much less efficient. The maximum yield of 1O_2 was 0.09 ± 0.02 mol/mol H_2O_2 consumed and required pH 4 and 5 mM H_2O_2 . At higher pH, the 1O_2 production rapidly decreased. The yield at pH 7 was 0.0004 ± 0.0002 mol/mol H_2O_2 consumed. Enzyme inactivation was a major factor limiting the yield of 1O_2 with both Cl^- and Br^- . While the MPO- H_2O_2 -halide systems can efficiently pro-

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Biochemical Requirements for Singlet Oxygen Production by Purified Human Myeloperoxidase

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duce 1O_2 , the conditions required are not physiologic, which suggests that the chemiluminescence of the stimulated neutrophil does not derive from 1O_2 generated by a MPO mechanism.

Introduction

The human neutrophil exhibits a nonmitochondrial respiratory burst upon stimulation with either soluble agonists (e.g., phorbol myristate acetate) or phagocytosable particles. (1-3). The products of this burst are reduction products of molecular oxygen, including superoxide, its dismutation product, hydrogen peroxide (H_2O_2), and hydroxyl radical, or species with like reactivity (1-3). The microbicidal capacity and deleterious toxicity to host tissue of the oxidizing system of H_2O_2 , halide, and the lysosomal enzyme, myeloperoxidase (MPO),¹ have been extensively characterized (4, 5). Recent reports have emphasized that hypochlorous acid (HOCl) is an oxidizing mediator of this system (6-9), but whether another reactive species, singlet oxygen (1O_2), is also a mediator of the phagocyte's microbicidal and cytotoxic properties is unsettled (2, 10, 11). While there are hypothetical MPO-independent mechanisms for 1O_2 formation in the respiratory burst of the human neutrophil (12), the MPO- H_2O_2 -chloride ion (Cl^-) system is the most likely source of any 1O_2 that is generated by stimulated neutrophils (13, 14). However, the question of 1O_2 production by neutrophils or by the MPO- H_2O_2 - Cl^- system is still unresolved, because the methods used in previous studies could not distinguish between 1O_2 and other oxidizing species (15-18). In particular, 1O_2 traps and quenchers used in those studies have been shown to react with HOCl or Cl_2 . The most convincing negative study for 1O_2 production was that of Foote et al. (19) using the specific 1O_2 trap, cholesterol.

Attempts to demonstrate 1O_2 production by searching for

1. Abbreviations used in this paper: CPO, chloroperoxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; 1O_2 , singlet oxygen.

its emission at 634 and 703 nm have been unsuccessful because the low intensity of any ${}^1\text{O}_2$ emission present is obscured by chemiluminescence from other sources (16, 20). The emission band at 1,268 nm, however, is directly proportional to the ${}^1\text{O}_2$ concentration (21). At the low concentrations of ${}^1\text{O}_2$ potentially present in the human neutrophil, the 1,268-nm band would be several orders of magnitude more intense than the dimole bands (17). Using newly developed infrared spectrophotometers that are highly sensitive to 1,268-nm radiation, efficient ${}^1\text{O}_2$ production by lactoperoxidase (LPO) and chloroperoxidase (CPO) has recently been reported (22–25). Emission at this wavelength appears to be a sensitive and specific test for ${}^1\text{O}_2$. We have now applied this methodology to determine if ${}^1\text{O}_2$ is produced by a purified human MPO system.

Methods

MPO was isolated from diisopropyl fluorophosphate (Sigma Chemical Co., St. Louis, MO)-treated human neutrophils that were disrupted by nitrogen cavitation (26). The fraction, which contains azurophilic granules, was harvested in Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradients as previously described (26), and the contents of this fraction were solubilized in 3% hexadecyltrimethylammonium bromide (Sigma Chemical Co.) and chromatographed over CM-Sepharose (Pharmacia Fine Chemicals) (applied in 0.1 M ammonium bicarbonate, pH 8.0, and eluted with 0.6 M (NH_4) HCO₃, pH 8.0) and Sephadryl S-200 (Pharmacia Fine Chemicals) (in 0.6 M (NH_4) HCO₃, pH 8.0) in a modification of the method of Andersen et al. (27). The A₄₃₀/A₂₈₀ = 0.83, and the protein demonstrates characteristic α and β dimeric bands of 64,000 and 15,000 mol wt on 10% electrophoresed sodium dodecyl sulfate (Bio-Rad Laboratories, Richmond, CA) polyacrylamide gels. The MPO activity, 180–240 U/mg, was defined using 4-aminoantipyrine as a hydrogen donor (28), and was assayed using an absorption coefficient of 89 mM⁻¹ cm⁻¹ at 430 nm (29). Heat-inactivated MPO was heated to 90°C for 15 min. Hypochlorous acid was distilled under reduced pressure from a 5.25% commercial solution (Clorox) that was acidified to pH 8 (30), and assayed at 292 nm using an absorption coefficient of 391 M⁻¹ cm⁻¹ (31). Solutions of hypobromous acid (HOBr), bromine, and tribromide anion, in equilibrium, were prepared by the addition of HOCl to buffers that contained excess Br⁻ (32). Due to the instability of these solutions, they were used immediately after their preparation. In the remainder of this report, HOBr will be used to denote the total concentration of all oxidized bromine species. The bromide concentration of purified human neutrophils, prepared by methods previously described (26), was measured by the formation of gold tribromide in acidic conditions from gold chloride (33). Cells suspended in phosphate-buffered saline (PBS) at a concentration of $8 \times 10^7/\text{ml}$ were sonicated at 4°C and then centrifuged at 30,000 g for 30 min. The pellets were again suspended in PBS and were assayed with the 30,000 g supernatants in duplicate. Calculations were based on a cell volume of 200 cu μm (34). Hydrogen peroxide was diluted from a 30% stabilized stock solution (superoxol, J. T. Baker, Phillipsburg, NJ) immediately before an experiment. Stock solutions were assayed iodometrically (35). Deuterium oxide (${}^2\text{H}_2\text{O}$), which was obtained from J. T. Baker, had an isotopic purity of 99.75%. Histidine was obtained from Sigma Chemical Co. All other inorganic chemicals were reagent grade and water was glass distilled.

Measurement of infrared chemiluminescence. The chemilumines-

cence spectrometer has been described in detail previously (22). Spectral analysis was carried out with a set of seven interference filters that were obtained from Pomfret Research Optics Inc., Stamford, CT. The filters had bandwidths of 50 nm and center wavelengths of 1,070, 1,170, 1,268, 1,377, 1,475, 1,580 and 1,680 nm. Emission measurements were corrected for the detector response and transmission of each filter. For kinetic experiments, the integral of the emission intensity over the entire reaction period was reported. An estimate of the amount of ${}^1\text{O}_2$ produced was obtained by comparing the emission integral of the system under study with that of the H₂O₂ plus HOBr reaction or the H₂O₂ plus HOCl reaction (24).

MPO systems. The MPO-H₂O₂-halide systems were studied at 25°C. Most experiments were performed in aqueous buffers that contained 98% ${}^2\text{H}_2\text{O}$. In these experiments, the p²H was calculated by subtracting 0.4 from the reading obtained with a glass electrode (36). Buffers were 100 mM sodium phosphate (p²H, 3, 6, 7, 8) or 100 mM sodium acetate (p²H, 4, 5), each containing the desired concentration of NaCl or NaBr. MPO, in 1.5 ml buffer contained in a dark adapted test tube, was placed in the spectrophotometer. The reaction was initiated by the rapid injection of an additional 1.5 ml of buffer containing H₂O₂. For calibration of the ${}^1\text{O}_2$ production, 1.5 ml of buffer containing HOBr or HOCl was placed in the spectrophotometer and the reaction initiated by the injection of an equal volume of buffer that contained H₂O₂.

Results

Evidence for ${}^1\text{O}_2$ production by MPO. Chemiluminescence at 1,268 nm was detected in the MPO catalyzed oxidation of both Cl⁻ and Br⁻. Recorder tracings of the time course of the emission under various conditions are shown in Fig. 1. While the intensity of the light with Br⁻ was comparable to that seen with LPO and CPO, the intensity with Cl⁻ was one to two

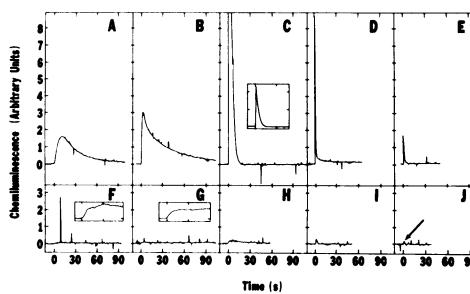


Figure 1. Time course of 1268-nm chemiluminescence in the MPO-H₂O₂-halide systems. Recordings were made with an amplifier time constant of 0.1 s. All experiments were done in 98% ${}^2\text{H}_2\text{O}$ with 100 mM sodium acetate (p²H 4, 5) or sodium phosphate (p²H 3, 6, 7) buffers, 1 mM H₂O₂, and 110 nM MPO. Top row had 10 mM NaBr; bottom row had 100 mM NaCl. *A*, p²H 3; *B*, p²H 4; *C*, p²H 5 (Inset shows signal reduced by a factor of 10. Time scale and amplifier time constant were unchanged.); *D*, p²H 6; *E*, p²H 7; *F*, p²H 3 (Sharp peak was noise spike. The observed signal appeared to be a prolonged shift in the base line shown more clearly in the inset with gain increased by a factor of 10 and the amplifier time constant increased to 10 s.); *G*, p²H 4 (inset as in *F*); *H*, p²H 5; *I*, p²H 6; and *J*, p²H 7.

orders of magnitude lower (22, 24). For this reason, most experiments were done in $^2\text{H}_2\text{O}$, which enhanced the intensity of the light by a factor of 30, due to the longer half-life of ${}^1\text{O}_2$ in $^2\text{H}_2\text{O}$.

There was substantial evidence to support the assignment of the observed infrared emission to enzymatically generated ${}^1\text{O}_2$. As shown in Table I, the emission consisted of a single band with a spectral distribution that was identical to that of ${}^1\text{O}_2$. No band was detected at 1,570–1,670 nm as reported by Khan (37) for CPO and catalase. The emission was inhibited by the ${}^1\text{O}_2$ quenchers, histidine, and azide, and was 29 ± 5 times greater in $^2\text{H}_2\text{O}$ than in H_2O (conditions were pH 5, 100 mM sodium acetate, 1 mM H_2O_2 , 10 mM NaBr, and 110 nM MPO). Enzyme activity was required for light production, since no emission was detected with heat-inactivated MPO or with the addition of azide or cyanide (Table II). The observed emission was not due to a reaction between HOBr and MPO. Replacement of H_2O_2 with HOBr produced only 0.004 ± 0.001 as much light (conditions were pH 5, 100 mM sodium acetate, 10 mM NaBr, 110 nM MPO, 1 mM H_2O_2 , or HOBr).

MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ system: kinetics and ${}^1\text{O}_2$ yield. Infrared emission was measured under a variety of conditions to optimize the ${}^1\text{O}_2$ production (Tables III and IV, Figs. 2 and 3). The mechanism proposed by Allen for ${}^1\text{O}_2$ generation by MPO, $(\text{H}_2\text{O}_2 + \text{H}^+ + \text{Br}^- \xrightarrow{\text{MPO}} \text{HOBr} + \text{H}_2\text{O}) (\text{H}_2\text{O}_2 + \text{HOBr} \rightarrow \text{H}_2\text{O} + \text{H}^+ + \text{Br}^- + {}^1\text{O}_2 (^1\Delta_g))$, predicts that 1 mol of ${}^1\text{O}_2$ will be produced for each 2 mol of H_2O_2 consumed (13). The chemiluminescence integral under optimal conditions (H_2O solvent, pH 5, 100 mM sodium acetate, 100 mM NaBr, 1 mM H_2O_2 , and 430 nM MPO) was compared with the

Table I. Spectral Analysis of the Infrared Chemiluminescence in the MPO- H_2O_2 -Halide Systems Compared to that of Singlet Oxygen Produced in the H_2O_2 Plus HOCl Reaction

Filter	Chemiluminescence*		
	MPO		
<i>nm</i>			
1,070	-0.002 ± 0.001	0.02 ± 0.01	0.000 ± 0.002
1,170	-0.001 ± 0.001	0.01 ± 0.01	0.003 ± 0.003
1,268	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.02
1,377	0.77 ± 0.01	0.66 ± 0.03	0.59 ± 0.01
1,475	0.12 ± 0.003	0.16 ± 0.04	0.14 ± 0.02
1,580	0.014 ± 0.004	0.01 ± 0.02	0.03 ± 0.004
1,680	-0.001 ± 0.003	0.02 ± 0.02	0.014 ± 0.002

* Each system was normalized so that the emission through the 1,268-nm filter was 1.00.

† 98% $^2\text{H}_2\text{O}$, pH 5, 100 mM sodium acetate, 10 mM NaBr, 1 mM H_2O_2 , and 44 nM MPO.

§ 98% $^2\text{H}_2\text{O}$, pH 5, 100 mM sodium acetate, 100 mM NaCl, 2 mM H_2O_2 , and 74 nM MPO.

|| 98% $^2\text{H}_2\text{O}$, pH 5, 100 mM sodium acetate, 100 mM NaCl, 0.5 mM H_2O_2 , and 0.5 mM HOCl.

Table II. Effect of Enzyme Inhibitors and ${}^1\text{O}_2$ Quenchers on 1,268-nm Chemiluminescence in the MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ System

Sample*	Relative chemiluminescence
Control	1.0 ± 0.1
Heat-inactivated enzyme	0.00 ± 0.01
1.5 mM KCN	0.00 ± 0.01
1.5 mM NaN_3	-0.01 ± 0.01
1.5 mM histidine	0.04 ± 0.01

* 98% $^2\text{H}_2\text{O}$, pH 5.0, 100 mM sodium acetate, 1 mM H_2O_2 , 10 mM NaBr, and 48 nM MPO.

emission integral of the H_2O_2 plus HOBr reaction. The validity and limitations of this calibration procedure have been the subject of a past study (24). The MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ system produced $83 \pm 5\%$ of the chemiluminescence that was predicted by the Allen mechanism.

As shown in Fig. 1 and Table III, the emission integral decreased at $\text{p}^2\text{H} > 5$. In Fig. 1 D, the emission was of high intensity but short duration, which suggests that enzyme inactivation was a major factor limiting the yield of ${}^1\text{O}_2$. Consistent with this hypothesis was the fact that the injection of a second aliquot of enzyme produced a second emission spike. Also, as seen in Tables III and IV, the chemiluminescence integral/ (H_2O_2) was increased by raising the enzyme concentration or by lowering the H_2O_2 concentration. Higher NaBr concentrations also favored more efficient production of ${}^1\text{O}_2$ at higher p^2H .

Bromide was quantitated in sonicated neutrophils to determine the physiologic availability of this halide. Free bromide (that is, the concentration in the cell – 30,000-g pellet) was undetectable, while that associated with the 30,000 g precipitable constituents was 6.6 ± 2.2 mM (mean \pm SD, $n = 4$). Presumably the Br^- associated with proteins is not available for MPO-catalyzed oxidations, but its function in this system could not be further characterized due to limitation of 1268-nm chemiluminescence sensitivity.

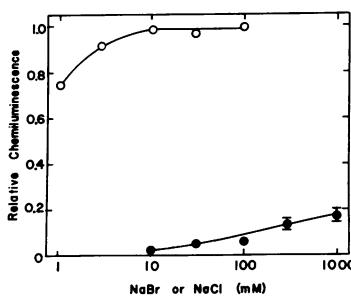


Figure 2. Effect of halide concentration on 1268-nm emission in the MPO- $\text{H}_2\text{O}_2\text{-Cl}^-$ and MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ systems. Conditions were 98% $^2\text{H}_2\text{O}$, pH 5, 100 mM sodium acetate, and 1 mM H_2O_2 . Enzyme concentrations were 44 nM for the MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ system (○), and 89 nM for the Cl^- system (●). Emission integrals in both systems were normalized to the maximum value seen in the MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ system. Error bars are not shown when the SEM is less than the size of the symbol.

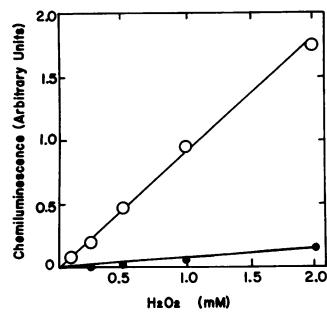


Figure 3. Effect of H_2O_2 concentration on the 1268-nm emission in the Cl^- and the MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ systems. Conditions were 98% $^2\text{H}_2\text{O}$, p^2H 5, and 100 mM sodium acetate. ○, MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ system, 44 nM MPO, and 10 mM NaBr; ●, Cl^- system, 89 nM MPO, and 100 mM NaCl. The size of the symbol indicates the SEM.

Cl^- system, kinetics, and $^1\text{O}_2$ yield. For the conditions studied, $^1\text{O}_2$ production by the Cl^- system was always inefficient. As shown in Table III, the maximum emission integral occurred at p^2H 4 with 5 mM H_2O_2 . Unfortunately, any chemiluminescence produced at H_2O_2 concentrations of 0.25 mM or less was below the detection limit of the spectrophotometer, so efficient production of $^1\text{O}_2$ at these low H_2O_2 concentrations cannot be excluded.

Enzyme inactivation was a major factor limiting the yield of $^1\text{O}_2$. At p^2H 6 and 7, the emission was of short duration, similar to the MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ system. Injection of a second aliquot of enzyme produced a second light spike. The behavior of the system was complex, however, since the emission integral did not increase with increasing enzyme concentrations.

At p^2H 3 and 4, the emission was of low intensity and long duration, consistent with low enzyme activity, which resisted rapid inactivation. The intensity was not limited by the rate of the H_2O_2 plus HOCl reaction, since the emission intensity of this reaction (0.5 mM H_2O_2 plus 0.5 mM HOCl) was one to two orders of magnitude higher than that seen with MPO (1 mM H_2O_2 , data not shown). The kinetic behavior of the system under these conditions is complex. As seen in Fig. 4, the onset of the emission was delayed by several seconds.

The addition of a second aliquot of enzyme during the period of prolonged light production abolished the emission for several seconds, which was followed by the return of chemiluminescence of lower intensity.

Discussion

The putative role of singlet oxygen in the respiratory burst of the human neutrophil has remained elusive due to the inability to reliably assess its quantitative production (2). Previous attempts to trace its generation by traps and quenchers have been discredited on the basis of the nonspecificity of the reactive profile (15–18). Chemiluminescence in the visible spectrum is both nonspecific and relatively insensitive. A previous study measuring radioactive cholesterol derivatives concluded that $^1\text{O}_2$, if produced at all, was a quantitatively insignificant product of the respiratory burst (19). In this study, we have examined chemiluminescence at 1,268 nm, a characteristic emission band of $^1\text{O}_2$, of a purified human MPO system to define the conditions and quantity of $^1\text{O}_2$ production. The validity and limitations of this method have been discussed previously (25). With the use of the purified MPO systems, we have determined conditions under which $^1\text{O}_2$ is generated, and that only at low acid pH with high H_2O_2 concentrations or at high bromide ion concentrations is this reaction favored.

The role of a Br^- -dependent MPO mechanism for $^1\text{O}_2$ production in stimulated neutrophils remains to be elucidated. The concentrations of free Br^- required for $^1\text{O}_2$ production are not found in the neutrophil (our results), and make this pathway a problematic source for $^1\text{O}_2$ production. (The Br^- associated with the 30,000-g neutrophil particulate fraction is ~60-fold greater than in whole blood (38), but it remains unassigned to a particular compartment or protein.) Because of technical limitations of signal to noise ratios, we have thus far been unable to confirm 1,268-nm chemiluminescence in stimulated neutrophils arising from the MPO- Br^- system. The

Table III. Effect of p^2H on 1268-nm Chemiluminescence in the MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ and MPO- $\text{H}_2\text{O}_2\text{-Cl}^-$ Systems

p^2H						
	3		4		5	
H_2O_2	Halide	(Chemiluminescence integral)/ H_2O_2 *				
mM	mM					
1	10, NaBr	0.53±0.01	0.90±0.05	0.91±0.03	0.14±0.004	0.026±0.001
0.1	10, NaBr		0.84±0.08	0.60±0.02	0.63±0.03	0.31±0.01
1	100, NaBr			1.00±0.01	0.24±0.01	0.04±0.002
0.1	100, NaBr				0.96±0.02	0.76±0.05
10	100, NaCl		0.10±0.01			
5	100, NaCl		0.22±0.05			
1	100, NaCl	0.12±0.02‡	0.09±0.03‡	0.04±0.005	0.014±0.003	0.001±0.0004
						-0.000±0.002

* The emission integral is expressed as a fraction of that seen in the MPO- $\text{H}_2\text{O}_2\text{-Br}^-$ system at p^2H 5, 1 mM H_2O_2 , and 100 mM NaBr. The MPO concentration was 110 nM. Experiments were done in 98% $^2\text{H}_2\text{O}$ with 100 mM sodium acetate (p^2H 4, 5) or 100 mM sodium phosphate (p^2H 3, 6, 7, 8). ‡ Estimated from the peak intensity and half-life of the chemiluminescence.

Table IV. Effect of Enzyme Concentration on the 1268-nm Chemiluminescence in the MPO-H₂O₂-Br⁻ and the Cl⁻ Systems

MPO nM	Chemiluminescence integral*			
	MPO-H ₂ O ₂ -Br ⁻ ‡		Cl ⁻ §	
	pH 5	pH 6	pH 5	pH 6
11	0.87±0.02			
22	0.96±0.01		0.12±0.01	
44	1.00±0.01	0.018±0.001	0.11±0.003	
89		0.037±0.003	0.07±0.01	0.003±0.001
180		0.084±0.003		0.004±0.001
370				0.005±0.001

* 98% ³H₂O, 100 mM sodium acetate, pH 5, or 100 mM sodium phosphate, pH 6, and 1 mM H₂O₂. The integral is expressed as a fraction of the emission integral of the MPO-H₂O₂-Br⁻ system at pH 5 and 44 nM enzyme.

† 10 mM NaBr.

§ 100 mM NaCl.

low pH at which Cl⁻ might efficiently serve as the halide source is not attained, even in the phagocytic vacuole, in the same time course as the chemiluminescent response of the phagocytosing neutrophil (20, 39–41); this Cl⁻ pathway then is an unlikely source for ¹O₂ in stimulated neutrophils.

The MPO-H₂O₂-Br⁻ system produced ¹O₂ in near stoichiometric yield, which is similar to the behavior of LPO and CPO (24, 25). However, in contrast to CPO, which also efficiently produced ¹O₂ with Cl⁻ as a substrate, the production of ¹O₂ by the Cl⁻ system was always inefficient (24). Enzyme inactivation by HOCl or chlorine (Cl₂) was a major factor limiting the yield of ¹O₂. As previously described in studies of MPO kinetics (42), the inactivation process is complex and depends

on the product of the H⁺ concentration (H⁺) and the Cl⁻ concentration, (Cl⁻). A high (H⁺) (Cl⁻) product reduces the HOCl concentration to which the MPO is exposed for three reasons. First, as demonstrated by Andrews and Krinsky (43), a high (H⁺) (Cl⁻) product will inhibit the MPO activity by raising the K_m for H₂O₂. This accounts, in part, for the low intensity of the emission under these conditions, since the enzymatic oxidation of Cl⁻ is the rate limiting step in the ¹O₂ production. Second, the rate of the H₂O₂ plus HOCl reaction is proportional to the (H⁺) (Cl⁻) product and consequently limits the accumulation of HOCl (30). Finally, a high (H⁺) (Cl⁻) product shifts the equilibrium between HOCl and Cl₂ toward Cl₂ (32). The reaction of Cl₂ with MPO may be less destructive than HOCl.

In our studies, when the (H⁺) (Cl⁻) product was low, for example at pH 6 and 100 mM Cl⁻, there was rapid and complete enzyme inactivation and the chemiluminescence was of short duration. Lowering the pH or raising the NaCl concentration at pH 5 resulted in prolonged low level emission (data not shown for high NaCl concentrations). Under these conditions, the onset of the emission was delayed and the light production was temporarily inhibited by the addition of fresh enzyme. One explanation for these observations is that the initial HOCl produced is consumed by highly reactive functional groups on the MPO. When these groups are fully saturated, a partially inactivated enzyme continues to make HOCl which reacts with H₂O₂ to produce ¹O₂. In the MPO-H₂O₂-Br⁻ system, enzyme inactivation is less significant. To some degree, this is due to the fact that HOBr is a weaker oxidizing agent than HOCl. The extremely rapid rate of the H₂O₂ plus HOBr reaction compared to the H₂O₂ plus HOCl reaction may be a more important factor, however, since this limits the HOBr concentration to a small fraction of the HOCl concentration in these systems (30, 44).

Based on pH and halide requirements of ¹O₂ production by purified MPO, it is unlikely that ¹O₂ is generated physiologically in the respiratory burst through a MPO mechanism. Although MPO is a potential source of ¹O₂, its production optimally occurs at nonphysiologic pH with nonphysiologic concentrations of free bromide. Compared to the MPO-H₂O₂-Br⁻ system, the Cl⁻ system is a poor source of ¹O₂, largely because of the much more rapid rate of the H₂O₂ plus HOBr reaction relative to the H₂O₂ plus HOCl reaction. This difference in reaction rates is likely to be even more important in the intact neutrophil, where the hypohalous acid can react with a high concentration of biomolecules (6, 7, 15, 45). Based on these studies, the physiologic production of ¹O₂ by an MPO-mediated system appears unlikely.

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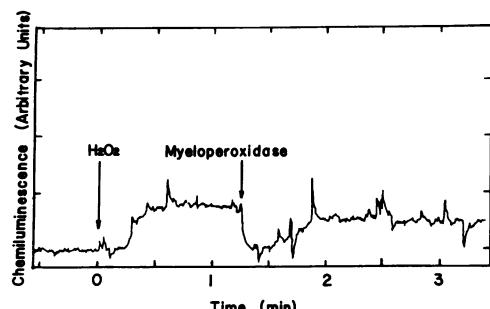


Figure 4. The disappearance of 1268-nm chemiluminescence in the Cl⁻ system caused by the addition of fresh MPO. Conditions were 98% ³H₂O, pH 4, 100 mM sodium acetate, 100 mM NaCl, 1 mM H₂O₂, and 89 nM MPO. 1 ml of buffer that contained MPO was placed in the spectrometer. The reaction was initiated by the injection of 1 ml of buffer that contained H₂O₂. Then an additional 1 ml of buffer that contained MPO was added. The amplifier time constant was 1 s.

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