MYELOPEROXIDASE AND SINGLET OXYGEN: A REAPPRAISAL

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Received 19 June 1978

1. Introduction

The concept that singlet oxygen may be an important physiological product of myeloperoxidase activity [1] has grown from the original observation by Allen that the myeloperoxidase-chloride (or bromide)-hydrogen peroxide system is chemiluminescent [2]. Allen postulated that hypochlorite, generated by the known activity of myeloperoxidase [3], reacts with excess peroxide to form singlet oxygen [4]. Apparent confirmation of singlet oxygen formation by Rosen and Klebanoff [5] was based on three pieces of evidence: (i) the system (MPO-Cl^--H_2O_2) converted diphenyl furan to *cis* dibenzoyl ethylene; (ii) this conversion was inhibited by singlet oxygen quenchers and traps; and (iii) the chemical conversion was enhanced in D_2O. However, these workers also reported that (iv) chloride ion increased the yield of the conversion; and (v) that hydrogen peroxide inhibited the conversion, whether or not it was effected by the enzymatic system, or by inorganic HOCl. It was then proposed that singlet oxygen arose in the enzymatic system not by the reaction sequence proposed by Allen [4], but spontaneously from HOCl [5]. Subsequently, O'Brien and coworkers [6], claimed that the lactoperoxidase-bromide-hydrogen peroxide system generated singlet oxygen by the reactions proposed by Allen [4]. Their conclusion was based on the findings that (i) the system (lactoperoxidase-bromide-peroxide) evolved oxygen; (ii) the system converted diphenylisobenzofuran to *o*-dibenzoylbenzene; (iii) the chemical conversion was inhibited by singlet oxygen quenchers or traps; (iv) chemiluminescence was observed, and was depressed by singlet oxygen traps, and enhanced in D_2O. However, these workers also reported that (v) singlet oxygen traps did not inhibit oxygen evolution; this is somewhat contradictory, since a high yield of *o*-dibenzoylbenzene (with respect to peroxide) was implied by the data.

In this paper, we show that the conclusions of Rosen and Klebanoff [5] are open to serious question because the singlet oxygen traps used also react with HOCl; their data then support the view that the test system employed measured the formation of chlorine. We show that the conclusions of O'Brien and coworkers [6] are equally questionable primarily on the same basis, and because the long lived chemiluminescence monitored in peroxidase-halide-peroxide systems is due to a secondary non-enzymatic process, involving added protein, which can continue long after the peroxidase has ceased to function. In the absence of any evidence for singlet oxygen, we discuss its possible relevance to the function of myeloperoxidase.

Abbreviations: MPO, myeloperoxidase; DPIBF, 1,3-diphenylisobenzofuran; DABCO, 1,4-diazabicyclo[2,2,2]octane; DPF, 2,5-diphenylfuran

2. Materials and methods

Myeloperoxidase was purified to a RZ value greater than 0.8 [7] and dialysed against water before use.

Diphenylisobenzofuran was obtained from Aldrich, diphenylfuran from Pfaltz and Bauer and diazabicyclooctane from MCB. Ethyl hydrogen peroxide (Ferrosan, Sweden) was obtained from Gallard-Schlesinger, and taurine from Sigma.

Total chemiluminescence was monitored using a RCA 31034A (red sensitive) photomultiplier tube cooled to −40°C by a thermoelectric housing (Pacific

Photometrics, Emeryville, Ca.). The tube yields a dark count rate of 25–50/s when moisture is excluded from the system. Current pulses from the tube were converted to voltage pulses across a 10 KΩ load, and amplification and conversion to logic pulses was done with an Ortec 4890 preamp-amp SCA module (Ortec, Inc., Oak Ridge, Tenn.). The count rate was monitored with a TC 596 ratemeter (Tennelec, Inc., Oak Ridge, Tenn.) which also drove the strip-chart recorder. The reaction vessel was a 15 ml vial equipped with a magnetic stirrer. Additions were made into the light-tight system via syringes attached to microbore tubing.

Chlorine (hypochlorite) was measured as previously described [3]. Optical experiments were performed using a Zeiss DMR-21 scanning instrument.

Details of individual experiments appear in the figure legends.

3. Results and discussion

3.1. *Chemiluminescence*

Figure 1 shows that the repeated injection of myeloperoxidase into a medium containing peroxide (50 mM) and chloride results in repeated bursts of chemiluminescence (only the decay of which is resolved) and an increasingly intense long-lived chemiluminescence. Essentially the same result was obtained when bromide ion was included. Omission of halide greatly reduced the response; chlorite-inactivated myeloperoxidase, or serum albumin, produced no chemiluminescent response. Chemiluminescence (both phases) was also reduced when taurine, which reacts with HOCl [9] was included (fig.1b). These data confirm previous observations showing that chemiluminescence from the system is dependent on the catalytic activity of myeloperoxidase in generating HOCl. The biphasic nature of the chemiluminescence is of interest, since it was also observed previously [2,5,6].

The addition of cyanide to the system after the first phase did not reduce the chemiluminescence emission during the second phase, showing that continued enzymatic activity is not occurring during this phase; similarly, the addition of taurine did not reduce the chemiluminescence, showing that free HOCl is not responsible for second-phase chemiluminescence. Figure 1c shows that 50 mM taurine did not completely scavenge HOCl in competition with the reaction with H_2O_2, and, however, that the rate of decomposition of HOCl under the conditions is far too rapid to account for the slow secondary

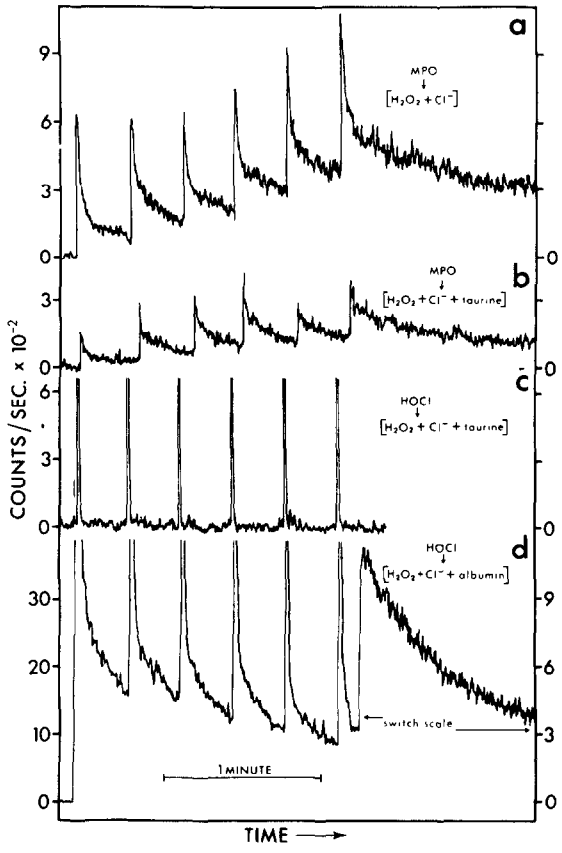

Fig.1. Chemiluminescent response to multiple additions of HOCl and myeloperoxidase. In (a), 0.2 ml (2.8 nmol) additions of myeloperoxidase (in H_2O) were made, at approximately 20-s intervals, to a rapidly stirred solution (10 ml) containing 0.1 M sodium acetate, 0.1 M sodium chloride and 50 mM hydrogen peroxide (pH 5.0). In (b), the conditions were identical, except that the solution contained 50 mM taurine. In (c) 50 μl additions 5.0 mM HOCl were made to the buffer as in (a). In (d) the buffer contained 1.2 mg/ml serum albumin. Each rapid upward deflection is caused by the addition of the indicated component; the essentials of each experiment are indicated in the figure. The light emission was monitored as described in methods. The instrument settings were identical for each experiment except for (d) where the high initial intensity of chemiluminescence necessitated a decrease in sensitivity.

phase seen in fig.1a and 1b. Thus, the second phase cannot be attributed to continued, low catalytic activity (HOCl generation) or to the slow decomposition of free HOCl formed during the first phase. No second phase chemiluminescence is observed in the non-enzymatic system (fig.1c).

An intense second phase chemiluminescence was however produced when a protein (chlorite-inactivated MPO, serum albumin, or lysozyme) was included in the inorganic system (fig.1d). Similarly, the presence of protein in the peroxidase system enhanced the second phase chemiluminescence. We conclude that the second phase of chemiluminescence is a reflection of the decay of unstable products of protein oxidation, the latter being produced during the first phase while the peroxidase is active. The nature of this chemiluminescence is under study.

3.2. *Chemical conversion*

Diphenyl furan is known to be oxidized to cis-dibenzoyl ethylene at the expense of Cl_2 [10]. Amino acids are known to rapidly form chloramines with HOCl [9]. Carotene is known to chemically quench halogens. It was suspected that the other singlet oxygen reagents used by Rosen and Klebanoff and O'Brien and coworkers could also react with halogens.

Solutions (20–100 μM) of chlorine water or bromine water were found to be quenched (no longer O-tolidine reactive) following the addition of diphenyl furan [5,6], bilirubin [5], methionine [6], histidine [6], or diazabicyclooctane [5,6] (examples, fig.2a). Carotene [5] was bleached by HOCl (fig.2b), and by the enzymatic system.

More importantly, diphenylisobenzofuran, the primary singlet oxygen indicator used by O'Brien and coworkers [6] also underwent oxidation at the expense of HOCl or HOBr. Figure 3 shows the ethyl hydrogen peroxide dependent oxidation of diphenyl-isobenzofuran by myeloperoxidase, proceeding at the rate of 2.21 nmol s^{-1} under the conditions used.

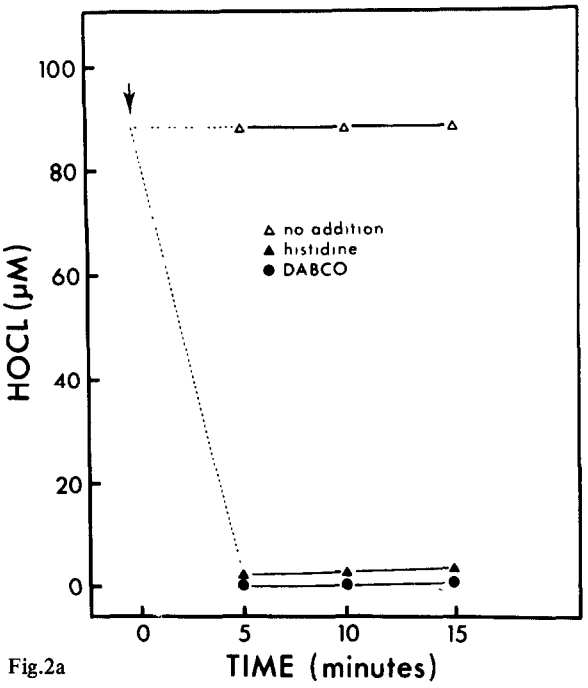

Fig.2a

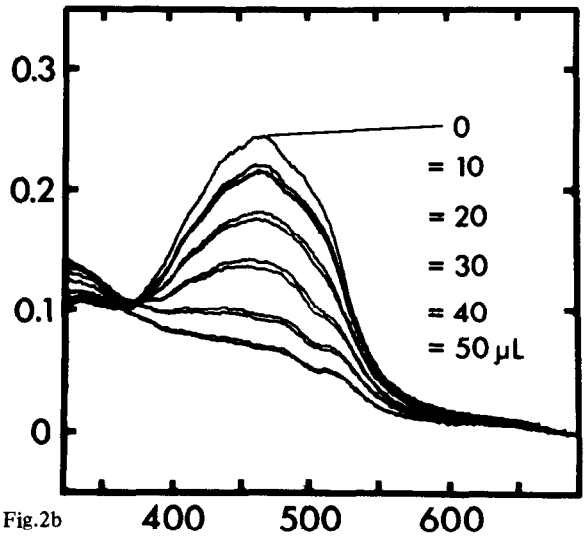

Fig.2b

Fig.2. (a) Solution stability of hypochlorite and its quenching by histidine or diazabicyclooctane (DABCO). The final concentrations (in 1.0 ml) were, hypochlorite 88 μM (176 μequiv./l); sodium acetate (pH 4.5), 0.1 M; sodium chloride, 0.1 M; diazabicyclooctane, 100 μM; histidine, 170 μM. The additions of the reagents, diazabicyclooctane (●), histidine (△), and water (▲) were made at $t = 0$. The solutions were incubated 5, 10 and 15 min, and assayed for chlorine using the O-tolidine reagent. (b) Bleaching of β-carotene by hypochlorite. The reaction mixture (1.0 ml) contained, 2.1 μM β-carotene in 0.1 M sodium acetate (pH 4.5) containing 0.1 M NaCl. 10 μl additions 90 μM hypochlorite were made and the optical spectrum scanned immediately after the addition and again after 1 min.

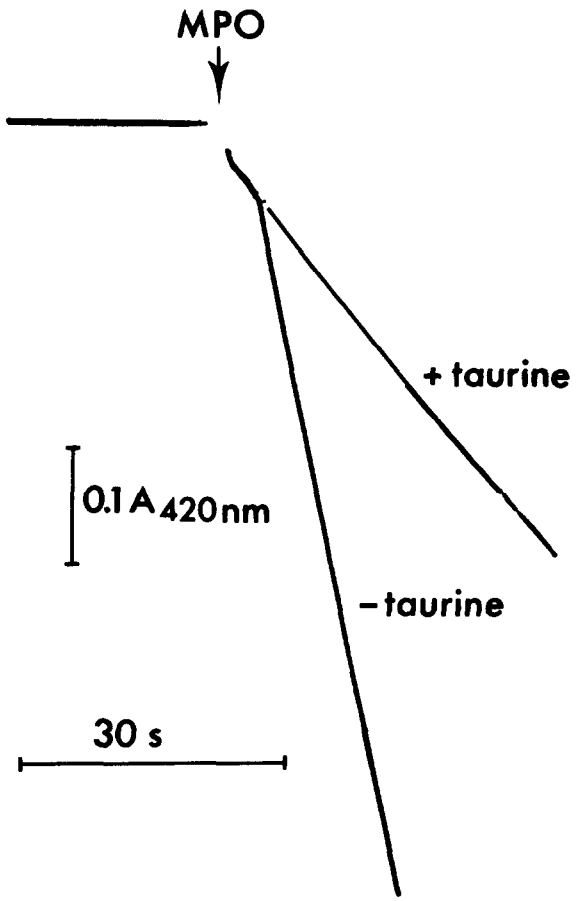

Fig.3. Myeloperoxidase catalysed oxidation of diphenylisobenzofuran with ethyl hydrogen peroxide as oxidant. The reaction mixture contained, in 1.0 ml 52 nmol diphenylisobenzofuran, 100 μmol sodium acetate (pH 4.5), 100 μmol sodium chloride, 1.3 μmol ethyl hydrogen peroxide; 140 pmol myeloperoxidase, 0.1% Triton X-100, and 50 μmole taurine, when present. The reaction was initiated by the addition of myeloperoxidase. Temperature, 26°C.

Taurine (50 mM) reduced the rate by 70%, equivalent to the trapping of 1.55 nmol (s^{-1}) of the species which oxidizes diphenylisobenzofuran. The rate at which taurine was chlorinated, as measured by the formation of taurine chloramine at 254 nm [13] (measured in the absence of diphenylisobenzofuran) was 1.45 nmol s^{-1}. This data allows a mechanism of inhibition of diphenylisobenzofuran oxidation to be formulated, in which HOCl is the relevant species, and is trapped by taurine. Previously conclusions were based solely on the presumed specificity of the traps used. It is also notable that the generation of HOCl, as measured by taurine chlorination, is tightly coupled to diphenylisobenzofuran oxidation. This coupling could not be expected if singlet oxygen were the oxidizing species, because the efficiency of singlet oxygen oxidations in aqueous media is very low.

4. Discussion

The idea has emerged that singlet oxygen may play an important role in host defense, both in the oxidative reactions of the respiratory burst, and as an important product of myeloperoxidase activity [1,2,4–6]. In this paper we have presented data which call into question the methodology for assaying singlet oxygen in peroxidase-halide-peroxide systems. The data immediately render the previous conclusions [5,6] unjustified. The conversion of diphenyl furan to cis-dibenzoyl ethylene by the MPO-Cl^--H_2O_2 system was due to the generation of Cl_2, not singlet oxygen, by the following summary. (i) The conversion occurs at the expense of Cl_2 [10] which is synthesized by MPO [3]; (ii) the conversion was stimulated by Cl^-, which favors Cl_2 in the chlorine ⇌ hypochlorite equilibrium [5]; (iii) the conversion was inhibited by hypochlorite reactive compounds (the presumed singlet oxygen quenchers); (iv) the conversion was inhibited by H_2O_2 (another hypochlorite-reactive compound) [5]; (v) insufficient enhancement in D_2O was observed to conclude that singlet O_2 was involved [5]; (vi) under conditions similar to those used by Klebanoff and Rosen, β-carotene was bleached by the enzymatic system; bleaching is a characteristic of the chemical reaction of carotene with chlorine, but not of the electronic quenching of singlet oxygen. Further, singlet oxygen is not known to arise spontaneously from HOCl, but is known to be generated from the reaction of H_2O_2 with HOCl [11]. Since H_2O_2 inhibited the enzymatic conversion [5], we conclude that conditions which might favor singlet oxygen formation are detrimental to the observed chemical conversion. This is hardly surprising, since singlet oxgyen can relax to triplet oxygen thereby becoming ineffective, while HOCl is stable in water over the time period used (fig.2).

A dismissal of the conclusion of O'Brien and

coworkers [6] is also indicated, and not only because of the conflict with the data of Rosen and Klebanoff [5]. Primarily, the chemical conversion data are susceptible to the same interpretation made above, with diazabicyclooctane and the amino acids [6] inhibiting the oxidation of diphenylisobenzofuran by scavenging HOBr, not singlet oxygen. Secondly, when our chemiluminescence data are considered, it becomes clear that (i) O'Brien and coworkers based their conclusions on the second phase chemiluminescence, which involves the peroxidase protein; and (ii) that the presumed singlet oxygen quenchers were quenching HOBr during the time the peroxidase was active. Thus, by adding taurine to the MPO-Cl^--H_2O_2 system, we were able to simulate the effect of methionine [6]; methionine also quenches HOCl (above). In addition, the statement that singlet oxygen traps did not influence oxygen evolution [6] is in conflict with the idea that they inhibited diphenylisobenzofuran oxidation by trapping singlet oxygen [6] since the traps used were indeed traps, not electronic quenchers.

We have also shown that diphenylisobenzofuran oxidation proceeds at the expense of an ethyl hydrogen peroxide-supported HOCl generation, and that the inhibition by taurine is compatible with its functioning as an HOCl trap. Since all the effective quenchers cited by O'Brien were HOCl- (and HOBr) reactive, and since it is clear that ethyl hydrogen peroxide cannot support singlet oxygen formation, we can conclude that the system measures HOCl, and that the system of O'Brien measured the formation of HOBr [6].

The data presented here show that previous work has failed to rule out free HOCl [3] as directly responsible for the presumed singlet-oxygen specific reactions. There remain two further considerations. If, as was originally proposed [4], singlet oxygen arises from a reaction of cell-generated H_2O_2 with myeloperoxidase-generated HOCl, what is the likely consequence of this reaction? Since the data of Rosen and Klebanoff [5] indicate that the reaction of H_2O_2 and HOCl is dissipative with respect to the oxidizing potential of HOCl, it is difficult to attach functional significance to this process. Secondly, what is the likelihood of such a process being of significance in the phagocytic vacuole? The way in which chemiluminescent experiments have been performed (quite apart from the assumptions of the origin of the emitted light), presuppose the presence of mM concentrations of H_2O_2 in the mileu of the peroxidase pool. However, it can just as easily be assumed that the hydrogen peroxide concentration is vanishingly low, an assumption more consistent with the high peroxidase content of the cell, and its catalatic activity [12]. While a full discourse of relevant functional properties of myeloperoxidase is beyond the scope of the present paper, we can also add that both H_2O_2 and HOCl (in the absence of an HOCl sink) saturate MPO, as the secondary peroxide compound, at μM concentrations [3,12,14], and the decomposition of this compound occurs at the expense of H_2O_2 [15]. While these observations do not rule out the accumulation of both H_2O_2 and HOCl in the phagocytic vacuole, they do suggest that the peroxidase is not functionally adapted to establish these conditions.

Acknowledgement

Supported by NIH Grant 10904-09.

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