SINGLET OXYGEN FORMATION DURING HEMOPROTEIN CATALYZED LIPID PEROXIDE DECOMPOSITION

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Summary The singlet oxygen trap diphenylfuran was rapidly oxidized to cis dibenzoylethylene during the decomposition of linoleic acid hydroperoxide catalyzed by ceric ions, methemoglobin or hematin. This conversion was enhanced in a deuterated medium and inhibited by other singlet oxygen quenchers or traps. The chemiluminescence accompanying the decomposition of the linoleic acid hydroperoxide was also markedly enhanced in a deuterated medium and inhibited by other singlet oxygen quenchers or traps. Antioxidants markedly inhibited these reactions. It is concluded that singlet oxygen is formed in substantial quantities during the metal catalyzed decomposition of linoleic acid hydroperoxide.

Singlet molecular oxygen ($^1O_2$) generated by dye-sensitized photooxygenation or by microwave discharge has been shown to modify amino acids, fatty acids and polysaccharides and nucleic acid bases and result in enzyme inactivation and lipid peroxidation (1). It has also been shown to be cytotoxic to tumour cells (2). Its formation in biological systems could therefore be of considerable biological consequence. Evidence for its formation by lipoxygenase has been presented (3) and convincingly refuted (4). Other investigators have suggested its formation by the NADPH dependent microsomal lipid peroxidation system (5,6) but this was refuted by others (7).

The ceric oxidation of sec-butyl hydroperoxide in benzene has previously been shown to cooxygenate singlet oxygen traps (8). A mechanism for singlet oxygen ($^1O_2$) formation involving self reaction of peroxy radicals was presented (8). Chemiluminescence, recently reported to be emitted during the decomposition of linoleic acid hydroperoxide by ceric ions was found to have an emission spectra different from the dimol chemiluminescence reported for a $OCl^- - H_2O_2$ aqueous

Abbreviations: LAHPO, Linoleic acid hydroperoxide; DPIBF, Diphenylisobenzofuran; DPF, Diphenylfuran; TLC, Thin layer chromatography; DABCO, 1,4 Diazobicyclo[2.2.2] octane.

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system known to produce singlet oxygen. Nonetheless they assigned the emission to singlet oxygen (9).

It was previously shown that linoleic acid hydroperoxide was rapidly decomposed by heme and hemoprotein to a range of products complex in nature due to the production of radical oxidizing species (10). In the following, unambiguous evidence is presented for singlet oxygen formation by this reaction under conditions similar to that which could occur physiologically.

Materials and Methods

The substances used in these studies were obtained from the following sources: 2,5-diphenylfuran, Eastman Organic Chemicals; trans 1,2-dibenzoylethylene, 1,3-diphenylisobenzofuran and O-dibenzoylbenzene, Aldrich Chemical Company. The substances were dissolved in acetone. Ceric ammonium sulfate was obtained from Sigma Chemical Company and was dissolved in 1 N HCl. Methemoglobin was obtained from Sigma.

Linoleic acid hydroperoxide (LAHPO) was prepared by the method of O'Brien (10)

Formation of O_2 Oxygen evolution from a reaction mixture containing 1 mM LAHPO, 1 mM ceric ammonium sulfate and 0.01 N HCl was monitored with a Clark type oxygen electrode at 20°C in a reaction vessel of 1.9 ml capacity. Oxygen uptake from a reaction mixture containing 50 mM Tris HCl buffer (pH 7.5), 100 μM LAHPO and 1 μM methemoglobin was monitored in a similar manner.

Chemiluminescence of the above reaction systems (5 ml) was monitored in the dark by photomultiplication methods using a Beckman Scintillation Counter (Model LS-233) operated in the out-of-coincidence mode (11). Counting vials were stored in the dark before use. The reaction was initiated by adding hydroperoxide. Scintillation counts were recorded every 10 seconds for the first minute and every thirty seconds thereafter. Systems containing D_2O were adjusted to a pH of 0.3 higher than H_2O containing systems so as to give an effective pD the same as the pH of the H_2O containing systems.

Oxidation of 1,3-diphenylisobenzofuran (DPIBF) DPIBF oxidation was measured spectrophotometrically by recording the decrease in absorbance at 420 nm of the reaction system (3 ml) containing 100 μM DPIBF after initiation of the reaction by the addition of hydroperoxide. Negligible oxidation occurred in the absence of hydroperoxide or methemoglobin.

Oxidation of diphenylfuran (DPF) Oxidation of DPF in a reaction system (3 ml) containing 3.3 μM DPF and 50 μM LAHPO was measured spectrofluorimetrically by recording the decrease in emission at 368 nm (slit 6 mμ) with excitation at 333 nm (slit 6 mμ) using a Perkin Elmer fluorescence spectrophotometer MPF 2A. The reaction was started with 0.5 μM methemoglobin. Negligible oxidation occurred in the absence of hydroperoxide or methemoglobin.

Thin layer chromatographic analysis of diphenylfuran and products The aqueous systems were extracted with 1 to 2 ml of chloroform/ml of reaction system at the end of each incubation period. The two phase system was vigorously mixed for approximately 15 seconds, then centrifuged for 10 minutes at 2,000 g and the chloroform layer was transferred as completely as possible to clean tubes. The chloroform was then removed by a flow of N_2 gas. The chloroform-soluble residue was transferred totally to thin layer plates (Silica Gel G) with small

TABLE I: The self reaction of linoleate peroxy radicals

The reaction medium contained linoleic acid hydroperoxide and ceric chloride as described in Methods.

	O_2 released (n moles)	Chemiluminescence c.p.m. x 10^{-3} at 20 sec.
Control	147	460
+ 0.1 mM histidine	141	46
+ 0.1 mM methionine	133	230
+ 0.1 mM nordihydroguaiaretic acid	65	313
+ 0.2 mM nordihydroguaiaretic acid	15	61
D_2O medium	130	990

volumes of chloroform. The plates were developed in the dark for one hour in a solvent system consisting of heptane-dioxane (3/1), air dried, and then examined under UV light or after exposure to iodine vapour.

Thin layer chromatographic analysis of diphenylisobenzofuran and products Prior to use, diphenylisobenzofuran (DPIBF) was purified by TLC on Silica Gel plates using benzene as the solvent. The purified DPIBF was added to the reaction mixture. The chloroform-soluble residue was extracted from the aqueous system and run on TLC as described above using benzene as the solvent.

RESULTS

The properties of the chemiluminescence accompanying the oxidation of linoleic acid hydroperoxide by ceric ions is shown in Table I. Evidence suggesting that $^1O_2$ is responsible was the marked inhibition by histidine and methionine known to be singlet oxygen traps (12) and the marked enhancement in a deuterated medium in which $^1O_2$ has been shown to have a longer lifetime (13). The anti-oxidant nordihydroguiaretic acid also markedly inhibited the chemiluminescence

TABLE II: Singlet oxygen formation during methemoglobin catalyzed linoleic acid hydroperoxide decomposition.

The reaction medium contained 0.1 mM linoleic acid hydroperoxide and 1 μM methemoglobin in 50 mM Tris HCl buffer (pH 7.5) for oxygen uptake and chemiluminescence measurements. DPIBF oxidation was followed at 420 nm with 100 μM DPIBF in the medium. The medium for DPF oxidation contained 3.3 μM DPF, 50 μM LAHPO and 0.5 μM methemoglobin. Methods are described in the experimental section.

	O_2 uptake nmoles/min.	Chemiluminescence c.p.m. x 10^{-3} at 20 sec.	DPF nmoles oxidized in 20 sec.	DPIBF nmoles/min
Control	137	736	4.6	120
+ 20 mM DABCO	134	1615	2.2	110
+ 1 mM azide	109	149	1.7	111
+ 10 mM histidine	70	227	2.7	95
+ 50 μM bilirubin	100	290	-	-
+ 50 μM nordihydroguaiaretic acid	62	35	0.4	11
+ 50 μM butylated hydroxyanisole	64	61	0.2	13
+ 100 μM DPIBF	423	-	-	-
+ 100 μM DPF	108	605	-	-
D_2O medium	148	1402	7.1	95
no hydroperoxide	17	23	0.2	6

as would be expected if self reaction of the peroxy radicals were responsible for the singlet oxygen formation. Oxygen electrode studies showed that oxygen was released by reactions of the peroxy radicals during the first minute but was followed by a slower oxygen uptake. From Table I it can be seen that

histidine and methionine however did not affect appreciably the amount of oxygen released indicating that they did not affect the peroxy radicals or their formation. Furthermore oxygen release was less in a deuterated medium indicating that the lifetime of peroxy radicals or their rate of formation was not enhanced.

Chemiluminescence also accompanied the decomposition of linoleic acid hydroperoxide catalyzed by hematin or methemoglobin at pH 7.5 (Table II) and moreover was much greater than that observed with ceric ions. No chemiluminescence was observed during the decomposition of LAHPO catalyzed by manganous, cobaltous or cuprous ions or by glutathione. Evidence suggesting that $^1O_2$ is responsible for the chemiluminescence was the marked stimulation by 1,4 diazobicyclo[2.2.2 octane (DABCO), a $^1O_2$ quencher (14) which enhances dimol emission in an aqueous medium (15). The $^1O_2$ quenchers azide (16), bilirubin (17) and the $^1O_2$ traps diphenylfuran (6) and histidine (12) also inhibited the chemiluminescence. Chemiluminescence was markedly enhanced in a deuterated medium. Antioxidants markedly inhibited the chemiluminescence suggesting that peroxy radicals were involved in the $^1O_2$ formation. According to Howard and Ingold (8), tertiary peroxy radicals cannot react to form $^1O_2$. In confirmation of this no chemiluminescence was observed during the decomposition of cumene hydroperoxide or tert-butyl hydroperoxide catalyzed by hematin and methemoglobin. This is further evidence that the chemiluminescence can be attributed to $^1O_2$. No chemiluminescence was observed during the decomposition of H_2O_2 catalyzed by hematin or methemoglobin.

The only oxidation products observed in a reaction mixture containing linoleic acid hydroperoxide, methemoglobin and diphenylfuran corresponded to cis dibenzoethylene, a $^1O_2$ product (6). From Table II it can be seen that the rate of oxidation of diphenylfuran was inhibited by DABCO, histidine and azide A stoichiometry of 10.2 moles of hydroperoxide were required to oxidize one mole of diphenylfuran. The only reaction product observed in a reaction mixture containing 1,3 diphenylisobenzofuran (DPIBF) corresponded to dibenzoylbenzene, a $^1O_2$ product. However as shown in Table II, the oxidation of DPIBF when assayed

at 420 nm was not affected by histidine or methionine. Furthermore the reaction was slower in a deuterated medium. The ready inhibition by antioxidants indicate that the peroxy radicals may catalyze the autoxidation of DPIBF to dibenzoylbenzene (18).

In contrast to the reaction with ceric ions the methemoglobin catalyzed reaction results in oxygen uptake. From Table II it can be seen that oxygen uptake was inhibited by antioxidants but was not affected appreciably by the singlet oxygen quenchers or traps and indicates that their effects on chemiluminescence could not be attributed to effects on the rate of decomposition of linoleic acid hydroperoxide catalyzed by methemoglobin. Furthermore the marked enhancement of oxygen uptake by DPIBF confirms that DPIBF undergoes autoxidation in this system.

Singlet oxygen may be formed (8) by the following mechanism, originally proposed by Russell (19) for the concerted self reaction of secondary peroxy radicals: $2R(H)OO^{\cdot} \longrightarrow R(H)OOOOR(H) \longrightarrow R(H)OH + RO + {}^1O_2$. In the present study it is likely that ${}^1O_2$ production by this reaction explains the chemiluminescence and the cooxygenation of diphenylfuran that accompanies the decomposition of linoleic acid hydroperoxide catalyzed by hemoproteins or ceric salts. A ${}^1O_2$ yield of 10.2% was observed during the decomposition of hydroperoxide catalyzed by methemoglobin.

Most of the oxygen release during the decomposition of linoleic acid hydroperoxide by ceric ions however may be due to the following non-concerted radical pathway (20): $2R(H)OO^{\cdot} \longrightarrow R(H)OOOOR(H) \longrightarrow 2R(H)O^{\cdot} + O_2$. The formation of peroxy radicals during the decomposition of linoleic acid hydroperoxide catalyzed by methemoglobin probably involves the reaction of alkoxy radicals (21): $RO^{\cdot} + ROOH \longrightarrow ROO^{\cdot} + ROH$.
The formation of alkyl radicals by the scission of these alkoxy radicals would also explain the observed rapid oxygen uptake as the alkyl radicals react rapidly with oxygen and enter the autoxidative chain sequence again as peroxy radicals (22). Alternatively alkyl radicals could be formed by the abstraction of a

hydrogen atom from the hydroperoxide by the alkoxy radicals and lead to dihydroperoxide formation.

The formation of $^1O_2$ from the peroxy radicals may explain some of the controversy regarding whether $^1O_2$ is formed during microsomal lipid peroxidation. Thus Smith and Teng (7) using cholesterol as a $^1O_2$ trap found only products normally formed by free radical catalyzed autoxidation. Presumably the peroxy radicals react with cholesterol faster than does $^1O_2$. A similar explanation may exist for the lack of $^1O_2$ products obtained in a lipoxygenase reaction (4) in which tetraphenylcyclopentadienone was used as the trap.

In our studies, the $^1O_2$ trap, 1,3-diphenylisobenzofuran underwent autoxidation initiated by the peroxy radicals (18). The oxygenation was not therefore enhanced by a deuterated buffer or inhibited by $^1O_2$ quenchers or traps. Care must therefore be exercised in the choice of $^1O_2$ traps to demonstrate $^1O_2$ formation. The rapid formation of $^1O_2$ and peroxy radicals during lipid peroxide decomposition catalyzed by hemoproteins at a neutral pH should prove to be of considerable physiological relevance. The enzymic formation of $^1O_2$ has recently been demonstrated (23).

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