Evidence for the Formation of Ozone (or Ozone-Like Oxidants) by the Reaction of Singlet Oxygen with Amino Acids

George W. Wanjala, Arnold N. Onyango, David Abuga, Calvin Onyango, and Moses Makayoto

1Department of Food Science and Technology, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000 (00200), Nairobi, Kenya
2Kenya Industrial Research and Development Institute, P.O. Box 30650 (00100), Nairobi, Kenya

Correspondence should be addressed to Arnold N. Onyango; arnold.onyango@jkuat.ac.ke

Received 16 April 2018; Revised 14 August 2018; Accepted 19 August 2018; Published 13 September 2018

Academic Editor: Serkos A. Haroutounian

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Antibodies or some amino acids, namely, cysteine, methionine, histidine, and tryptophan, were previously reported to catalyse the conversion of singlet oxygen ($^1\text{O}_2$) to ozone ($\text{O}_3$). The originally proposed mechanism for such biological ozone formation was that antibodies or amino acids catalyse the oxidation of water molecules by singlet oxygen to yield dihydrogen trioxide (HOOOH) as a precursor of ozone and hydrogen peroxide ($\text{H}_2\text{O}_2$). However, because HOOOH readily decomposes to form water and singlet oxygen rather than ozone and hydrogen peroxide, an alternative hypothesis has been proposed; ozone is formed due to the reaction of singlet oxygen with amino acids to form polyoxidic amino acid derivatives as ozone precursors. Evidence in support of the latter hypothesis is presented in this article, in that in the presence of singlet oxygen, methionine sulfoxide ($\text{RS}(\text{O})\text{CH}_3$), an oxidation product of methionine ($\text{RSCH}_3$), was found to promote reactions that can best be attributed to the trioxidic anionic derivative $\text{RS}^+(\text{OOO}^-)\text{CH}_3$ or ozone.

1. Introduction

Ozone ($\text{O}_3$) is a highly reactive gas, which is mainly found in the Earth’s stratosphere, where it is formed through photolysis of an oxygen molecule ($\text{O}_2$) by solar radiation of below 242 nm, which splits $\text{O}_2$ into two oxygen atoms (O) (Equation (1)), followed by reaction of an oxygen atom with an oxygen molecule in a three-body reaction (Equation (2)), where the third body (M) is often $\text{N}_2$ or $\text{O}_2$ [1]:

$$\text{O}_2 \xrightleftharpoons{hv} \text{O} + \text{O} (\lambda < 242 \text{ nm}) \quad (1)$$

$$\text{O} + \text{O}_2 + \text{M} \rightarrow \text{O}_3 + \text{M} \quad (2)$$

Stratospheric ozone plays the vital role in protecting organisms on earth from the harmful effects of solar radiation of 240–320 nm [1]. Some ozone is also formed in the troposphere, mainly through photolysis of nitrogen dioxide ($\text{NO}_2$) to form nitric oxide (NO) and an oxygen atom, followed by reaction of the latter with $\text{O}_2$ according to Equation (2); volatile organic compounds such as car exhaust fumes contribute to formation of $\text{NO}_2$ [2]. Tropospheric ozone is regarded as a pollutant which is harmful to the respiratory system and contributes to the pathogenesis of insulin resistance, diabetes, and cardiovascular dysfunctions [3–6]. Paradoxically, ozone also finds application in alternative medicine, for example, in treatment of diabetic ulcers, as recently reviewed [7]. Interestingly, ozone (or an oxidant with the chemical signature of ozone) has also been reported to be generated in biological systems involving antibodies, amino acids, formaldehyde, neutrophils, and myeloperoxidase [8–12]. It has also been reported that endogenous ozone is a key player in atherogenesis or the killing of bacteria by neutrophils and some antibiotic compounds [9–12]. Such biological ozone formation requires singlet oxygen ($^1\text{O}_2$) and is catalysed by antibodies or the amino acids cysteine, methionine, histidine, or tryptophan [9, 10]. Myeloperoxidase (MPO) promotes the formation of singlet...
oxygen by catalysing the conversion of hydrogen peroxide ($H_2O_2$) to hypochlorous acid (HOCl), followed by a reaction of the latter with $H_2O_2$ (Equation (3)), and this may be the source of singlet oxygen for neutrophil-dependent, antibody-catalysed ozone production by neutrophils [11]. There are many other sources of singlet oxygen in vivo, as recently reviewed [13, 14]:

$$H_2O_2 \xrightarrow{MPO,Cl^{-}} HOCl \xrightarrow{H^+} \cdot O_2 + H_2O + Cl^{-} \quad (3)$$

Some of the evidence that was presented to support the antibody- or amino acid-catalysed ozone formation included the occurrence of three known ozone reactions, namely, the conversion of indigo carmine, 4-vinylbenzoic acid, or cholesterol to isatin sulfonate, 4-carboxybenzaldehyde, or 3β-hydroxy-5-oxo-5,6-seco-cholestan-6-al (secosterol A), respectively, when the mentioned reactants were incubated with antibodies or amino acids in the presence of singlet oxygen [8–10]. Although some doubts have been raised concerning the uniqueness of such reactions to ozone [15], no direct evidence against ozone formation has been demonstrated. On the other hand, the antibiotic effect of the oxidant generated by amino acids or antibodies in the presence of singlet oxygen was clearly shown to be distinct from singlet oxygen or hydrogen peroxide and to be more compatible with ozone or an ozonelike oxidant [9, 10]. Moreover, ozone produced in plant tissues has reportedly been detected directly by gas chromatography-mass spectrometry in the selective ion-monitoring mode (GC-MS-SIM) [3].

The mechanisms of biological ozone formation have not been firmly established although two pathways have been proposed. According to the first pathway, commonly referred to as the water-oxidation pathway, antibodies or amino acids catalyse the oxidation of water by singlet oxygen to form dihydrogen trioxide (HOOOH), followed by a not-so-well-defined decomposition of the latter to ozone and hydrogen peroxide [8–10]. However, HOOOH has been found to readily decompose to singlet oxygen and water, rather than hydrogen peroxide and ozone [16]. Hence, an alternative hypothesis was proposed, involving the reaction of amino acids with singlet oxygen to form oxidized amino acid derivatives, followed by further reaction of the latter to form organic zwitterionic polyoxidic derivatives which decompose to release ozone, as exemplified in the methionine 1-catalysed ozone formation via methionine persulfoxide 2, methionine sulfoxide 3, and a trioxynionic methionine derivative 4 (Scheme 1) [17]. Singlet oxygen might also react with methionine persulfoxide 2 to form ozone and methionine sulfoxide 3 via tetroxide intermediate 5 (Scheme 1). This mechanism is based on the fact that singlet oxygen is an electrophile and would thus react with the anionic oxygen atoms in compounds 2 and 3, and ozone release from intermediates 4 and 5 would be favoured because it results in formation of relatively stable neutral molecules 1 and 3. On the other hand, analogous ozone formation from HOOO$^-$, derived from HOOOH, would require the energetically unfavourable formation of a hydride anion (H). Some reactions involved in the decomposition of ozone by water (Equations (4)–(7)) [17–19] are also worthy of consideration in that the reactions of ozone with the hydroxide ion or hydroperoxide anion (Equations (4) and (6), respectively) are analogous to the reactions of singlet oxygen with compounds 2 and 3 in Scheme 1. It is possible that Equations (4) and (6) are reversible, with HO$_2^-$ and HO$_5^-$ being precursors of ozone, like compounds 4 and 5 in Scheme 1. However, as per Equation (7), HO$_5^-$ easily undergoes radical decomposition as well so that such radical decomposition may be more important under high ozone concentration. On the other hand, in tetroxianion 4, the positive charge on sulphur likely makes radical decomposition to form radical 6 and superoxide anion less favourable than conversion of 4 to methionine 1 and ozone or methionine sulfoxide 3 and singlet oxygen. In fact, the reaction of ozone with methionine 1 was previously found to yield singlet oxygen and methionine sulfoxide 3 [20, 21].

$$O_3^+ + OH \longrightarrow HO_4^- \quad (4)$$
$$HO_4^- \longrightarrow HO_2^- + O_2 \quad (5)$$
$$HO_2^- + O_3 \longrightarrow HO_5^- \quad (6)$$
$$HO_5^- \longrightarrow HO_2^- + O_3 \quad (7)$$

The present study is the first to directly test and show the role of an amino acid oxidation product, methionine sulf oxide 3 in the formation of ozone. Methionine sulfoxide, rather than any other amino acid oxidation product, was used because it is readily available.

### 2. Materials and Methods

#### 2.1. Reagents

Human myeloperoxidase (MPO), hydrogen peroxide ($H_2O_2$), potassium chloride (KCl), methionine, methionine sulfoxide, indigo carmine, isatin sulfonic acid, vinylbenzoic acid, 4-carboxybenzaldehyde, potassium orthophosphate buffer ($KH_2PO_4$), and acetonitrile were purchased from Sigma Aldrich.

#### 2.2. Conversion of Indigo Carmine to Isatin Sulfonate in the Presence of Singlet Oxygen and Methionine or Methionine Sulfoxide

Five units of MPO were dissolved in 1 mL of 100 mM potassium orthophosphate buffer (pH 7.4). 0.1 mL of this solution was mixed with 4.4 mL of 100 mM $KH_2PO_4$ (pH 7.4), 100 mM KCl, 100 μM $H_2O_2$, 150 μM indigo carmine, and 700 μM methionine or methionine sulfoxide. The mixture was incubated at 37°C for 1 hour. As a control, a similar reaction without methionine or methionine sulfoxide was done. The reaction mixture was subjected to HPLC on a reverse phase C18 column eluted with a solvent consisting of acetonitrile (30%) and 50 mM phosphate buffer (pH 7.4) (70%) containing 0.1% trichloroacetic acid. Isatin sulfonate and residual indigo carmine were identified by comparing their retention times with respective standards. Peak areas were converted to concentrations by comparison to an isatin sulfonate standard curve.
2.3. Conversion of Vinylbenzoic Acid to 4-Carboxybenzaldehyde in the Presence of Singlet Oxygen and Methionine or Methionine Sulfoxide. This was determined as described above for the conversion of indigo carmine to isatin sulfonate, except that, during the reaction, indigo carmine was replaced by 4-vinylbenzoic acid, and during HPLC, 4-vinylbenzoic acid and 4-carboxybenzaldehyde standards were used instead of indigo carmine and isatin sulfonate standards.

3. Results and Discussion

Singlet oxygen was generated by the myeloperoxidase-H$_2$O$_2$-chloride system [11, 22]. The conversion of indigo carmine to isatin sulfonate was found to occur in the control experiment as well as in the presence of methionine and methionine sulfoxide. The system containing methionine gave slightly lower yield than the control while the methionine sulfoxide system gave higher yield than the control (Table 1).

The conversion of indigo carmine to isatin sulfonate is not an ozone-specific reaction but can also be accomplished by singlet oxygen [9, 10]. Singlet oxygen is expected to convert indigo carmine 7 to dioxetane 8 that decomposes to form two molecules of isatin sulfonate 9 (Scheme 2). According to this scheme, each of the two molecules of isatin sulfonate incorporates an oxygen atom from singlet oxygen. For ozone-mediated conversion of indigo carmine to isatin sulfonate, however, there is incorporation of an oxygen atom from water, and such incorporation of water-derived oxygen atoms was previously confirmed in studies of antibody- or amino acid-catalysed ozone formation [9, 10]. In the present study, no attempt was made to determine the source of oxygen atoms in the isatin sulfonate molecules. The reduced isatin sulfonate formation in the presence of methionine might be partly due to some physical quenching of singlet oxygen by methionine [23]. On the other hand, higher formation of isatin sulfonate in the presence of methionine sulfoxide than in the control experiment is indicative of the involvement of another oxidant beside singlet oxygen, which in previous studies was reported to be ozone or an oxidant with the chemical signature of ozone [9, 10].

Incubation of 4-vinylbenzoic acid (10 in Scheme 3) in the myeloperoxidase-H$_2$O$_2$-chloride system led to minimal formation of 4-carboxybenzaldehyde, which is consistent with a previous finding that singlet oxygen does not convert 4-vinylbenzoic acid to 4-carboxybenzaldehyde [10]. This may be understood from the fact that reaction of vinyl aromatics with singlet oxygen more favourably proceeds through a [2 + 4] cycloaddition to form endoperoxides, rather than [2 + 2] cycloaddition to form dioxetanes [24]. Thus, the reaction of singlet oxygen with 4-vinylbenzoic acid

### Table 1: Yield of isatin sulfonate during the myeloperoxidase-catalysed generation of singlet oxygen in the presence of methionine or methionine sulfoxide.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Isatin sulfonate (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70</td>
</tr>
<tr>
<td>Methionine</td>
<td>64</td>
</tr>
<tr>
<td>Methionine sulfoxide</td>
<td>83</td>
</tr>
</tbody>
</table>

**Scheme 1**: Previously proposed pathway for methionine-catalysed ozone formation via methionine oxidation products such as methionine persulfoxide 2, methionine sulfoxide 3, and a trioxyanionic derivative 4 [17] and suggested possibility of ozone and methionine sulfoxide formation from persulfoxide 2 via tetroxide anion 5.

**Scheme 2**: Previously proposed pathway for methionine-catalysed ozone formation via methionine oxidation products such as methionine persulfoxide 2, methionine sulfoxide 3, and a trioxyanionic derivative 4 [17] and suggested possibility of ozone and methionine sulfoxide formation from persulfoxide 2 via tetroxide anion 5.

**Scheme 3**: Reactivation of vinyl aromatics with singlet oxygen.
should more readily generate, via a \([2 + 4]\) cycloaddition, endoperoxide 11, whose decomposition does not afford 4-carboxybenzaldehyde 12 (Scheme 3). On the other hand, methionine and methionine sulfoxide generated significant amounts of 4-carboxybenzaldehyde as illustrated in Figure 1, further supporting the involvement of an oxidant different from singlet oxygen. Ozone reacts by 1,3-dipolar cycloaddition with various unsaturated organic compounds [25, 26] and converts 4-vinyl benzoic acid to 4-carboxybenzaldehyde [9, 10, 27]. The finding that methionine sulfoxide (an oxidation product of methionine) promoted 4-carboxybenzaldehyde formation therefore supports the proposal that amino acids promote ozone formation by reacting with singlet oxygen, and the most plausible explanation for the methionine sulfoxide 3-mediated ozone formation is its further reaction with singlet oxygen to form trioxyanionic intermediate 4 (Scheme 1) as explained in Introduction.

Intermediates such as 4 and 5 might, in addition, directly react as ozonelike oxidants. For example, as postulated in Scheme 3, intermediate 5 might undergo a nucleophilic reaction with ozone to form a dioxetane-like intermediate 6. The reaction of 4-vinyl benzoic acid with ozone would result in the formation of 4-carboxybenzaldehyde 7, which might then react with ozone to form the dioxetane intermediate 8. This intermediate might then decompose to form the final product 9.

Figure 1: HPLC chromatograms of vinylbenzoic acid (a), 4-carboxybenzaldehyde (b), and the reaction mixture obtained by incubating vinylbenzoic acid and methionine sulfoxide with a singlet oxygen-generating myeloperoxidase system (c), showing some conversion of vinylbenzoic acid to 4-carboxybenzaldehyde. Incubating methionine with vinylbenzoic acid and the myeloperoxidase system gave a similar chromatogram.

Scheme 2: Mechanism of the singlet oxygen-mediated conversion of indigo carmine to isatin sulfonate via a dioxetane intermediate.
addition to 4-vinylbenzoic acid 10 to form carbanionic intermediate 13, which may convert via dioxetane 14 or ozonide 15; and the direct reaction of vinylbenzoic acid 10 with singlet oxygen to form peroxide 10 which does not produce 4-carboxybenzaldehyde 12.

4. Conclusion
Evidence has been presented that methionine sulfoxide, an oxidation product of methionine, reacts with singlet oxygen to form ozone or an ozonelike oxidant, thus supporting the hypothesis that biological ozone or ozonelike oxidant formation involves the sequential reaction of singlet oxygen with amino acids and amino acid oxidation products.

Data Availability
The data used to support the findings of this study are included within the article. Other data are available from the corresponding author upon request.

Conflicts of Interest
The authors declare that there are no conflicts of interest.
Acknowledgments
This work was funded by a grant from The World Academy of Sciences for the advancement of science in developing countries (TWAS) (14–075RG/CHE/AF/ACI; UNESCO FR:
324028586) to A. N. Onyango.

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