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OPINION PAPER

Alternatives to the 'water oxidation pathway' of biological ozone formation

Arnold N. Onyango [1]

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Abstract Recent studies have shown that ozone (O_3) is endogenously generated in living tissues, where it makes both positive and negative physiological contributions. A pathway for the formation of both O_3 and hydrogen peroxide (H_2O_2) was previously proposed, beginning with the antibody or amino acid-catalyzed oxidation of water by singlet oxygen ($^1O_2$) to form hydrogen trioxide (H_2O_3) as a key intermediate. A key pillar of this hypothesis is that some of the H_2O_2 molecules incorporate water-derived oxygen atoms. However, H_2O_3 decomposes extremely readily in water to form $^1O_2$ and water, rather than O_3 and H_2O_2. This article highlights key literature indicating that the oxidation of organic molecules such as the amino acids methionine, tryptophan, histidine, and cysteine by $^1O_2$ is involved in ozone formation. Based on this, an alternative hypothesis for ozone formation is developed involving a further reaction of singlet oxygen with various oxidized organic intermediates. H_2O_2 having water-derived oxygen atoms is subsequently formed during ozone decomposition in water by known reactions.

Keywords Ozone · Singlet oxygen · Hydrogen peroxide · Amino acid oxidation · Baeyer-Villiger oxidation

Introduction

Ozone gas (O_3, $^{\delta+}O{=}O{-}O^{\delta-}$) is an important component of the stratosphere where it protects organisms on earth from the most damaging wavelengths of solar radiation [1]. On the other hand, tropospheric ozone may be harmful to the respiratory system [1]. Nevertheless, ozone gas finds some application in alternative medicine [2] as well as in water and wastewater treatment [3].

Over a decade ago, Wentworth et al. [4] reported that antibodies catalyze ozone formation in the presence of water and singlet oxygen ($^1O_2$), partly based on the observed occurrence, under such conditions, of reactions that were thought to be unique to oxidation by ozone. These reactions included the conversion of indigo carmine to isatin sulfonic acid and the conversion of cholesterol to 3β-hydroxy-5-oxo-5,6-sechocholestan-6-al (secosterol aldehdye A) and 3β-hydroxy, 5β-hydroxy- B-norcholestane-6β-carboxaldehyde (secosterol aldehyde B). On the positive side, ozone generation in this manner contributes to bacterial killing by neutrophils [4]. Moreover, one of the best photodynamic therapy (PDT) strategies for cancer treatment involves the use of antibodies conjugated to photosensitizers [5], and the anticancer effect of this type of PDT may partly be due to antibody-catalyzed ozone generation upon irradiation of the photosensitizers and formation of $^1O_2$. On the other hand, the secosterol aldehydes produced by cholesterol ozonolysis potentially contribute to the pathogenesis of disorders such as atherosclerosis and Alzheimer's disease [6,7].

The concept of ozone generation in biological systems generated reasonable skepticism, and experimental results were obtained proving that conversion of indigo carmine to isatin sulfonic acid, or the formation of cholesterol secosterol aldehydes could also be mediated by oxidants other than ozone [8,9]. For example, it was demonstrated that cholesterol 5-hydroperoxide, obtained by the reaction of cholesterol with singlet oxygen, undergoes Hock cleavage to form the secosterol aldehydes [9]. However, the singlet oxygen-Hock cleavage pathway predominantly generates secosterol aldehyde B [7,9], while the ozonolysis pathway predominantly

✉ Arnold N. Onyango
arnold.onyango@jkuat.ac.ke

[1] Department of Food Science and Technology, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000, 00200 Nairobi, Kenya

generates secosterol aldehyde A [7]. The fact that the latter is the major secosterol aldehyde in atherosclerotic tissues [7] and is also produced in vitro by neutrophils [10] supports the occurrence of ozone-mediated cholesterol oxidation in vivo [11]. More recently, the formation of endogenous ozone by plant leaves was proved directly by GC-MS-SIM [1]. It was also reported that the antibiotic activity of a number of compounds including trans-resveratrol, salicylic acid, and cinnamic acid involves the endogenous formation of formaldehyde and ozone [2,12,13].

A pathway involving the oxidation of water by $^1O_2$ was proposed for the antibody-catalyzed ozone formation [4], mainly based on the fact that some hydrogen peroxide molecules (H_2O_2) formed under the same conditions incorporate oxygen atoms from water molecules [14]. However, this proposal, which assumes that the O_3 and H_2O_2 are products of the same pathway, has not been unequivocally proved.

Here, I reinterpret available literature and develop an alternative hypothesis for O_3 formation involving the oxidation and deoxidation of specific types of organic compounds. Pathways of methionine-, tryptophan-, and histidine-catalyzed ozone formation as well as a pathway of ozone formation during formaldehyde oxidation are proposed as examples of O_3 generation in this manner. A common feature of all the ozone-forming steps is that $^1O_2$ reacts as an electrophile with a nucleophilic oxygen atom in the immediate precursor molecule, whose structure is such that loss of an O_3 molecule results in co-formation of a non-charged product. I also propose that the decomposition of O_3 in water may be largely responsible for the production of H_2O_2 containing water-derived oxygen atoms.

The antibody/amino acid-catalyzed water oxidation pathway and the need for alternative pathways of O_3 formation

The antibody-catalyzed ozone formation occurs under conditions where H_2O_2 is also generated in a high yield of >500 molecules per antibody molecule [4,14]. Thus, O_3 and H_2O_2 were postulated to be formed by a common pathway, the antibody-catalyzed water oxidation pathway which begins with the reaction of $^1O_2$ with water to generate hydrogen trioxide (H_2O_3) as a key intermediate (Fig. 1) [4]. Two possible routes for the conversion of H_2O_3 to H_2O_2 and O_3 have been proposed and shown to be feasible based on theoretical calculations [15,16]. The first of these routes involves a reaction of H_2O_3 with $^1O_2$ while the other involves a reaction of the former with another molecule of H_2O_3 (Fig. 1). Notably, however, a third route for H_2O_3 decomposition involving its water catalyzed conversion to $^1O_2$ and water (Fig. 1) is well established both theoretically and experimentally [16]. In fact, H_2O_3 is extremely unstable (with a half-life of less than a second) under aqueous conditions, where it basically decomposes to H_2O and $^1O_2$ [16]. The antibody-catalyzed formation of O_3 and H_2O_2 was suggested to occur in a hydrophobic site where the H_2O_3 is shielded from water [14]. However, even in organic solvents, all attempts to unambiguously detect H_2O_2 and O_3 as products of the decomposition of H_2O_3 have failed [16].

The possible role of amino acid oxidation in the antibody-catalyzed formation of H_2O_2 as an alternative to the water oxidation pathway was discounted because of the consideration that, even if every photooxidizable amino acid residue (cysteine, methionine, histidine, tryptophan, and tyrosine) in

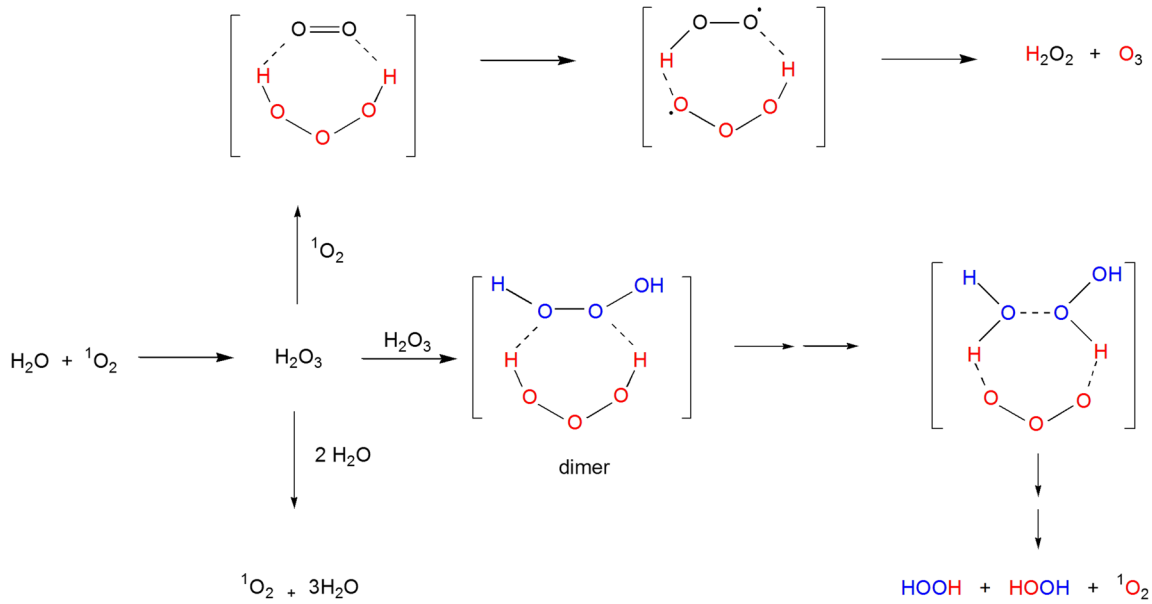

Fig. 1 The water oxidation pathway involving the initial formation of H_2O_3 followed by decomposition of the latter by (i) reaction with singlet oxygen to form H_2O_2 and O_3, (ii) reaction with another H_2O_3 molecule to form $2H_2O_2$ and $^1O_2$ with the intermediacy of ozone, or (iii) the water catalyzed decomposition to $^1O_2$ and H_2O [4,15,16]. The latter reaction is extremely facile under aqueous conditions [16]

an antibody molecule were consumed, this could not account for the >500 mole equivalents of H_2O_2 generated [14]. Curiously, however, Yamashita et al. [17] found that out of 19 amino acids tested (excluding tyrosine), the photooxidizable amino acids methionine, cysteine, histidine, and tryptophan catalyzed the formation of ozone in the presence of singlet oxygen, comparably to antibodies, and that peptides lacking these amino acids did not generate ozone. They postulated that the amino acids catalyzed ozone formation through the water oxidation pathway but did not propose how the amino acids performed the catalysis. However, a major role for H_2O_3 in ozone formation under these conditions is doubtful, even from the consideration that hydrophobic sites for its stabilization in amino acid solutions are unlikely to be present.

Hence, there is a need to explore alternative pathways for O_3 and H_2O_2 formation that do not involve H_2O_3 as the key intermediate.

Evidence that the oxidation of amino acids and other organic molecules may be important for ozone generation

The common feature of the four amino acids that catalyze ozone formation [17] is that they are photooxidizable [14], and numerous studies have documented their oxidation by singlet oxygen under physiologically relevant conditions, both in the free form and as components of proteins [18–32].

As components of proteins, the reactivity of these amino acids with $^1O_2$ greatly depends on their positions in the proteins, with residues exposed to solvent being oxidizable, while the less accessible residues remain unoxidized [20,21]. While only few amino acid residues in antibodies seem to get oxidized in the presence of singlet oxygen [22], such few residues might be sufficient for O_3 and H_2O_2 formation. For example, just one exposed tryptophan residue was found to contribute 50 % of H_2O_2 production by a monoclonal antibody [23]. To date, the oxidative modification of specific tryptophan, methionine, and histidine residues in antibodies and monoclonal antibodies by singlet oxygen has been reported [23–26]. Of particular interest is the recent discovery that $^1O_2$ mediates the formation of cross-linked histidine-histidine dimers [27] and that two identical conserved histidine residues in the highly flexible and solvent-accessible hinge region of an immunoglobulin G1 (IgG1) antibody undergo photooxidation to form such dimers [26].

Exposure of plant leaves to ozone causes damage to the leaves' photosystem 2 (PS II) [33]. PS II damage was also found to occur during light-induced oxidative stress in a process that involves $^1O_2$-mediated oxidation of specific tryptophan residues [29]. PS II damage under the latter conditions is consistent with endogenous ozone production by the leaves [1] through a process involving tryptophan oxidation. In a related study, exogenous histidine was found to promote oxygen uptake and damage to PS II of the cyanobacterium *Synechocystis* PCC 6803, and the increased oxygen uptake was attributed to 'chemical trapping' of singlet oxygen by histidine [30].

Tyihak et al. [12,13] more recently reported that compounds such as formaldehyde, cinnamic acid, and resveratrol were precursors of ozone. They postulated a formaldehyde/O_3 pathway in which formaldehyde reacts with H_2O_2 to generate activated formaldehyde and $^1O_2$, followed by participation of the latter in the water oxidation pathway to generate ozone [12,13]. However, in atmospheric chemistry, formaldehyde is known as an important ozone precursor by a mechanism involving its oxidation [34] and not merely the production of $^1O_2$.

Thus, it is worthwhile to consider pathways of $^1O_2$-mediated amino acid and formaldehyde oxidation, with the aim of identifying potential steps that could be involved in O_3 formation.

Suggested pathways of ozone formation via reactions of singlet oxygen with amino acids or formaldehyde

It was recently reported that various aliphatic and aromatic aldehydes react with $^1O_2$ to form the corresponding organic acids [35]. The initial reaction of the aldehyde with $^1O_2$ involves a hydride transfer and generates a peroxyacid, which undergoes a Baeyer-Villiger oxidation with a second aldehyde molecule to form two molecules of acid [35]. In the Baeyer-Villiger oxidation, the peroxyacid acts as a nucleophile while the aldehyde acts as an electrophile. Since $^1O_2$ is a good electrophile, it may plausibly compete with the aldehyde in the Baeyer-Villiger oxidation step. Hence, formaldehyde **1** may be converted via peroxyformic acid **2** to formic acid **3** and ozone (Fig. 2). Thus, the formaldehyde/O_3 pathway may involve formaldehyde reacting with H_2O_2 to produce $^1O_2$ [12,13], followed by reactions depicted in Fig. 2.

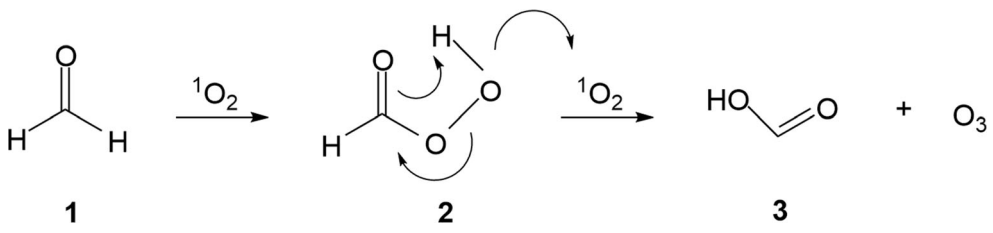

Fig. 2 Proposed mechanism of formation of ozone through reactions of formaldehyde (**1**) and singlet oxygen ($^1O_2$)

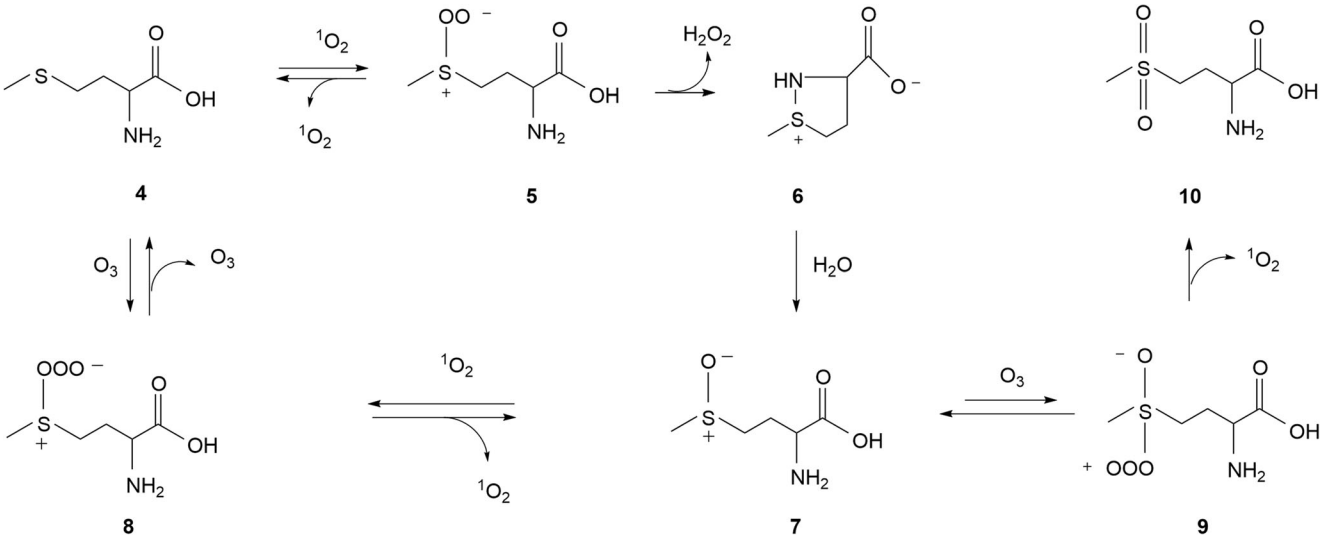

Fig. 3 Proposed pathway of O_3 formation via the successive reactions of methionine (**4**) and methionine sulfoxide (**7**) with singlet oxygen ($^1O_2$). The competing conversion of **7** to sulfone **10** normally occurs to a limited extent

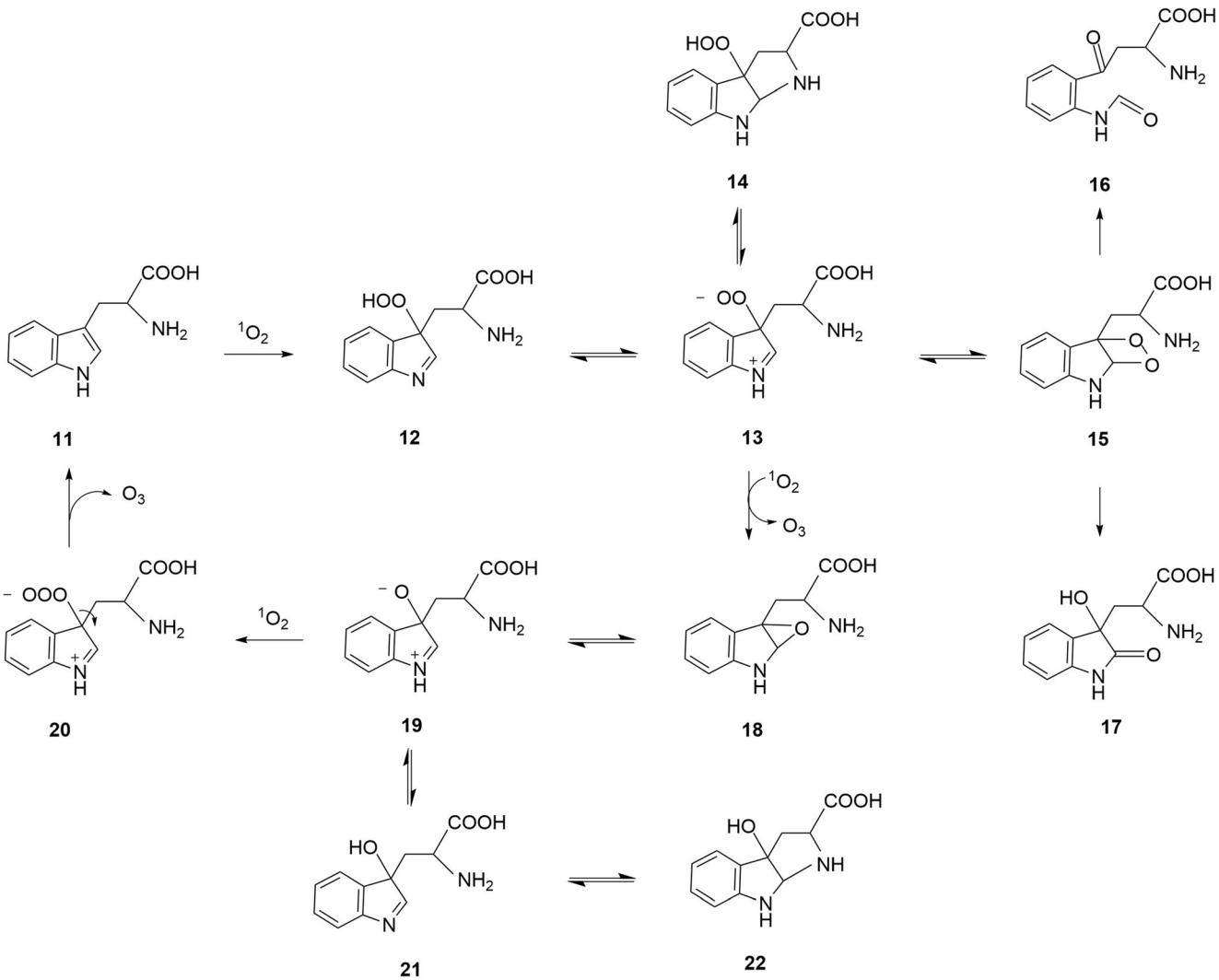

Fig. 4 Proposed pathways of O_3 formation via reactions of tryptophan **11** and its oxidation products with singlet oxygen ($^1O_2$)

Tomono et al. [36] found that decomposition of the pure $^1O_2$ generator, 1-methylnaphthalene-4-endoperoxide (MNPE) at 37 °C and pH 7.4 in the presence of cholesterol resulted in the formation of secosterol aldehyde B and a small amount of secosterol aldehyde A, and that addition of IgG resulted in a decrease in the former aldehyde and an increase in the latter. Increased formation of secosterol A in the presence of IgG is easily explained by the antibody-catalyzed ozone formation from $^1O_2$. On the other hand, formation of secosterol A even in the absence of IgG might be partly explained by secosterol aldehyde B reacting with singlet oxygen analogously to the ozone-producing reactions of formaldehyde with singlet oxygen.

Between pH 6 and 10, the reaction of methionine **4** with $^1O_2$ in water proceeds via persulfoxide **5** and the cyclic dehydromethionine **6** to form methionine sulfoxide **7** [36] (Fig. 3). Interestingly, under similar conditions, methionine **4** also reacts efficiently with O_3 to generate sulfoxide **7** and $^1O_2$ [37,38]. Since the reaction between H_2S and O_3 proceeds through an intermediate adduct H_2S-O_3 [39], the reaction of **4** with O_3 to form **7** may also proceed through a trioxysulfoxide adduct, **8** (Fig. 3). Although methionine sulfoxide **7** may be further oxidized irreversibly to form methionine sulfone, this reaction is usually unfavorable [40]. A possible mechanism for the latter reaction might begin with a nucleophilic attack of O_3 on the S atom of sulfoxide **7** to generate intermediate **9** whose decomposition affords the sulfone **10** (Fig. 3). Such ability of O_3 to react as a nucleophile has been previously reported [3]. On the other hand, it is conceivable that the reaction of $^1O_2$ at the nucleophilic oxygen of sulfoxide **7** can produce trisulfoxide adduct **8** which decomposes to O_3 and methionine **4**. This proposed reversibility of the reaction of methionine **4** and O_3 to form sulfoxide **7** and $^1O_2$ is indirectly supported by the fact that methionine sulfoxide reductases readily convert the sulfoxide **7** back to methionine [40,41]. The yield of O_3 during the proposed reaction of **7** and $^1O_2$ is expected to be good because ozone's co-product, **4**, will react with $^1O_2$ to regenerate reactant **7**. Likewise, the reported high yield of $^1O_2$ and methionine sulfoxide **7** from O_3 and methionine **4** should be due to the very short lifetime of $^1O_2$ (less than a second as compared to 4.8 min half-life of ozone in water) [37,38].

Figure 4 illustrates a suggested pathway for tryptophan-catalyzed ozone formation. First, tryptophan **11** reacts with singlet oxygen to form hydroperoxide **12** [18,28,32], which can exist in equilibrium with zwitterionic peroxide **13** and tricylic hydroperoxide **14**. The latter is a well-known tryptophan photooxidation product [31,32]. Zwitterionic peroxide **13** may cyclize to form dioxetane **15** whose decomposition

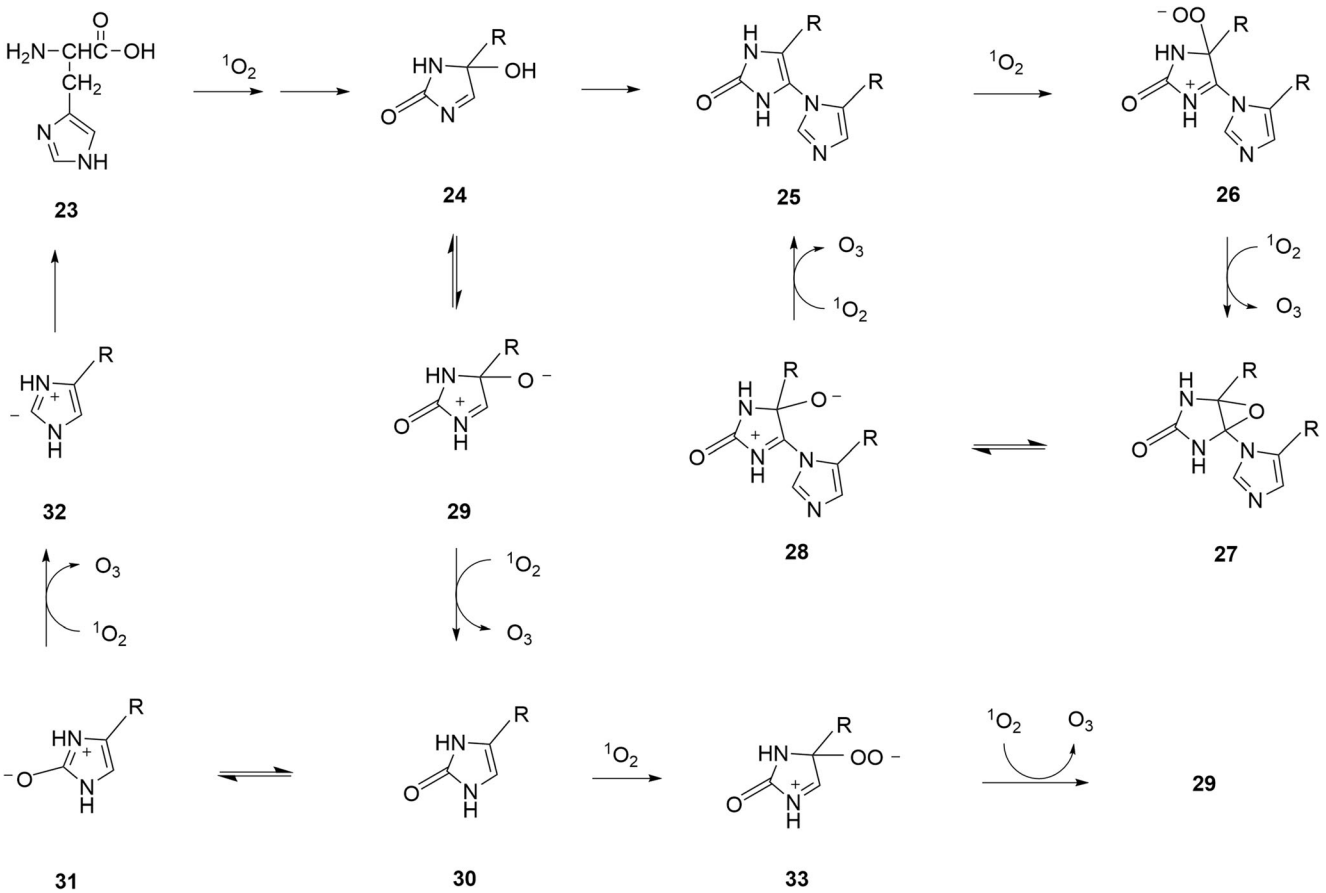

Fig. 5 Proposed pathways of O_3 formation via reactions of histidine **23** and its oxidation products with singlet oxygen ($^1O_2$)

affords N-formylkynurenine **16** [28,32] or dioxindolylalanine **17** [32]. Alternatively, I propose that peroxide **13** may react with $^1O_2$ to afford O_3 and epoxide **18** in a concerted reaction like the above proposed reaction of peroxyacid **2** with $^1O_2$ (Fig. 2). However, a stepwise reaction of $^1O_2$ and **13** via a zwitterionic tetroxide is not ruled out. Epoxide **18** can exist in equilibrium with zwitterionic oxide **19**, which may also react with $^1O_2$ to form ozone and regenerate tryptophan **11** via trioxyanion **20**. A concerted reaction between **19** and $^1O_2$, bypassing **20** is also a possibility. Compounds **18** and **19** can exist in equilibrium with isomeric alcohols **21** and **22**, which are known products [28,31,32].

Figure 5 illustrates some of the potential pathways involved in the histidine-catalyzed ozone formation. First, histidine **23** is converted to hydrated imidazolone **24** [18,27,28], which is a precursor of the histidine-histidine cross-linked dimer **25** [26] that has been detected in the hinge region of an IgG 1 [26]. A cyclic oxidation-deoxidation pathway of this dimer will lead to O_3 production via intermediates 26–28. Ozone formation may also occur during the conversion of compound **24** via zwitterionic oxide **29** to 2-oxohistidine **30**, which is a known product [26]. The latter may be converted back to histidine **23** via zwitterionic oxide **31** and vinylic anion **32**. Alternatively, **30** may be converted back to **29**, with O_3 formation, via zwitterionic peroxide **33**.

The pathways suggested in Figs. 4 and 5 for tryptophan and histidine-catalyzed ozone formation are not exhaustive, and other oxidized intermediates such as dioxindolylalanine **17** are also potential O_3 precursors.

Ozone decomposition in water may play a key role in the formation of H_2O_2 containing water-derived oxygen

The incorporation of oxygen atoms from water into H_2O_2 molecules in the presence of $^1O_2$ was considered as key evidence that water reacts with $^1O_2$ to form H_2O_3 according the water-oxidation pathway [14]. However, H_2O_2 containing water-derived oxygen may potentially be formed during the decomposition of O_3 by known reactions as depicted in Fig. 6. For example, when O_3 formation occurs during irradiation of antibodies, equation 1 is likely to be a major contributor of such H_2O_2 [42]. H_2O_2 that does not contain water-derived oxygen can be generated during the oxidation of methionine (Fig. 3) or by ozonolysis of tryptophan or histidine via α-

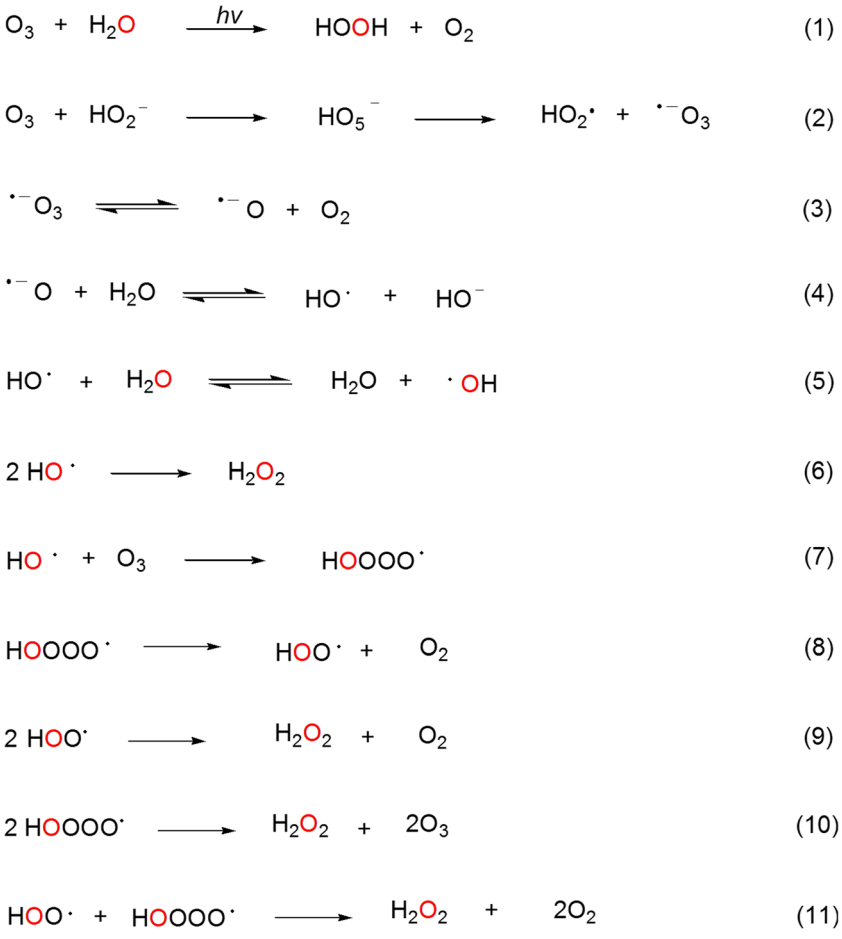

Fig. 6 Proposed formation of H_2O_2 containing water-derived oxygen atoms as a consequence of the decomposition of O_3

hydroxyhydroperoxide intermediates [43]. HO_2 anions derived from such H_2O_2 molecules can initiate O_3 decomposition and formation of hydroxyl radicals (OH) according to equations 2–4 [44]. Hydroxyl radicals undergo rapid hydrogen exchange with water molecules [45]. Thus, even if some hydroxyl radicals formed according to equation 4 have ozone-derived oxygen atoms, their hydrogen exchange with water molecules will generate hydroxyl radicals having water-derived oxygen according to equation 5. There is reasonable chance that such hydroxyl radicals will meet and react to form H_2O_2 (equation 6) [46]. Other reactions that would lead to formation of H_2O_2 with water-derived oxygen include equations 7–11 [47]. By such pathways therefore, the oxidation of water to form H_2O_2 may occur as a consequence of O_3 formation and decomposition, contrary to the previously proposed ozone formation as a consequence of water oxidation.

Concluding remarks

In the present article, potential pathways have been proposed for (i) the oxidation of aldehydes by $^1O_2$ to form acids and O_3 and (ii) the amino acid-catalyzed O_3 formation via various oxidation and deoxidation reactions. Theoretical and experimental efforts to confirm these pathways will be possible because potential key intermediates have been identified.

Antibodies may play an important role in ozone production by neutrophils [4]. While efforts to understand the finer details of the antibody-catalyzed O_3 formation have in the past focused on potential hydrophobic active sites for formation of H_2O_3 [14,22], more attention should now shift to mapping solvent accessible sites where methionine, histidine, tryptophan, cysteine, methionine, and disulfide bridges undergo oxidation, and how chemical modifications to these sites affect O_3 generation.

Based on the types of O_3-generating reactions suggested here, it will be possible to predict the potential of various other organic compounds to participate in or catalyze ozone formation. It will also be possible to synthesize new ozone-generating molecules, which may help to advance the use of ozone in various fields. For example, such molecules may serve as alternatives to the antibiotics currently used in crop protection.

Compounds such as 1-butylnaphthalene-4-propionate endoperoxide or 1-methylnaphthalene-4-propionate endoperoxide generate $^1O_2$ under physiological conditions in the dark [48,49]. The potential application of such compounds to generate ozone for treatment of various diseases remains to be explored. For example, the malaria parasite, *Plasmodium falciparum*, which easily develops drug resistance, has been shown to be inhibited by ozone [50] and, interestingly, it abundantly produces a histidine-rich protein [51] that might be a good catalyst of ozone formation in the presence of singlet oxygen.

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