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SPECIAL ISSUE

Vanadium Chloroperoxidases: The Missing Link in the Formation of Chlorinated Compounds and Chloroform in the Terrestrial Environment?

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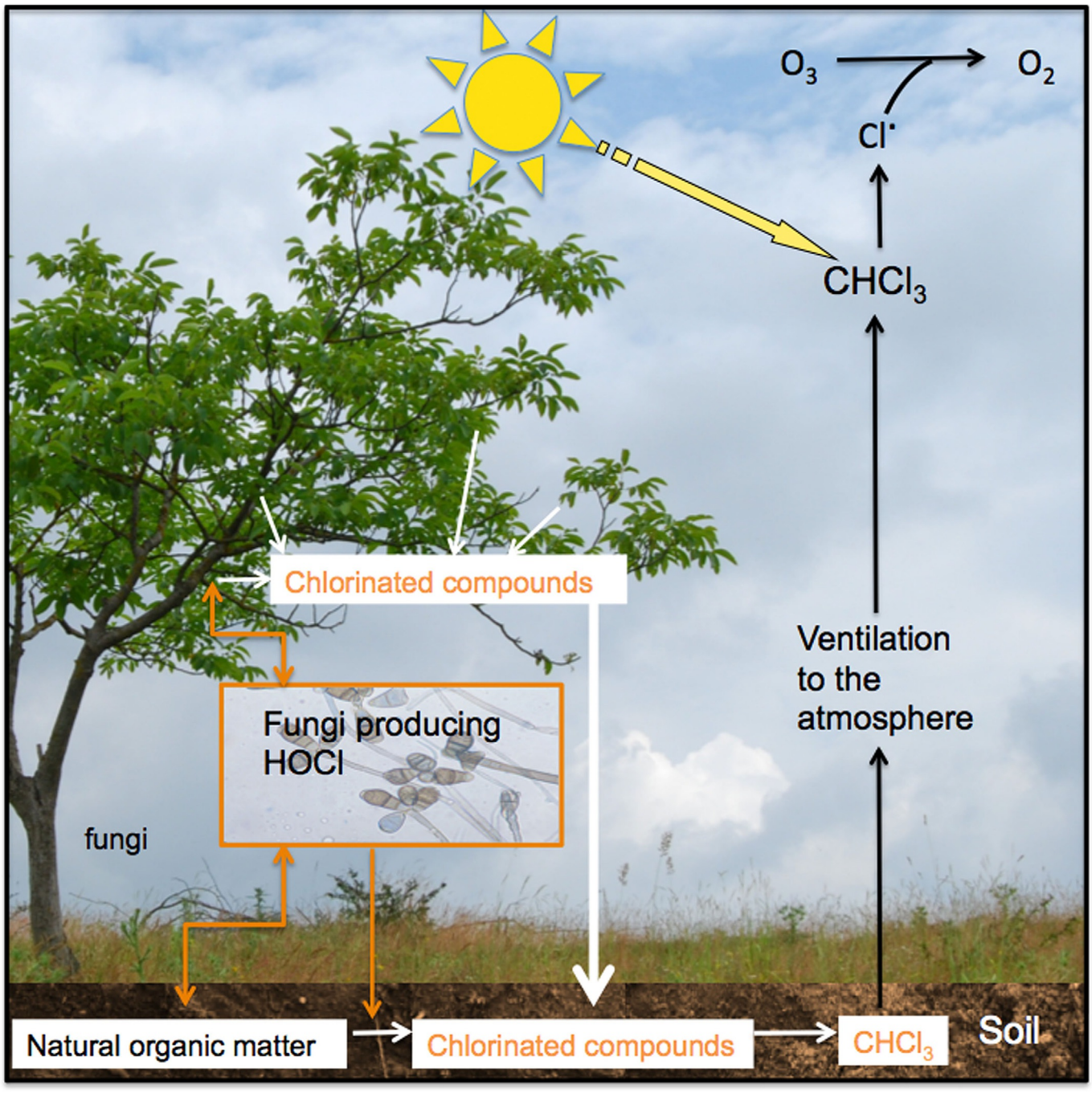

Abstract: It is well established that the majority of chlorinated organic substances found in the terrestrial environment are produced naturally. The presence of these compounds in soils is not limited to a single ecosystem. Natural chlorination is also a widespread phenomenon in grasslands and agricultural soils typical for unforested areas. These chlorinated compounds are formed from chlorination of natural organic matter consisting of very complex chemical structures, such as lignin. Chlorination of several lignin model compounds results in the intermediate formation of trichloroacetyl-containing compounds, which are also found in soils. These decay, in general, through a haloform-type reaction mechanism to $CHCl_3$. Upon release into the atmosphere, $CHCl_3$ will produce chlorine radicals through photolysis, which will, in turn, lead to natural depletion of ozone. There is evidence that fungal chloroperoxidases able to produce HOCl are involved in the chlorination of natural organic matter. The objective of this review is to clarify the role and source of the various chloroperoxidases involved in the natural formation of $CHCl_3$.

1. Introduction

Both marine and terrestrial ecosystems produce and release halogenated compounds. Brominated compounds dominate the marine environment[1,2a,b] and chlorinated organics are majorly produced on land[3] by chlorination of natural organic matter. This substrate consists of complex chemical structures, such as lignin. Several studies set up to investigate chlorination of lignin by using 1,3-dihydroxy aromatic model compounds and humic acid revealed intermediate formation of several trichloroacetyl-containing compounds.[4,5] These compounds, notably found in forest soils,[6] in general, decay to $CHCl_3$ through a haloform-type reaction mechanism favored under slightly alkaline conditions.[7] These data show that $CHCl_3$ may be considered as a natural compound in forest ecosystems.[8,9] Chlorination of dissolved organic material in freshwater by bleach also leads to the formation of $CHCl_3$ and other volatile metabolites;[10,11] a mechanism thus similar to natural chlorination. The presence of natural chlorinated organic compounds in soils and the formation of $CHCl_3$[8] is not limited to temperate forest ecosystems. An extensive study showed that natural chlorination was widespread and included grasslands and agricultural soils typical of unforested areas.[12] Boreal forest soils also emit $CHCl_3$ in similar amounts to that of more southern ecosystems.[13] Substantial emissions of $CHCl_3$ have also been reported for tundra soil in Alaska,[14] hypersaline sediments,[15] and sediments from a moderately acidic salt lake.[16] In temperate conifer ecosystems, $CHCl_3$ production is associated with microbial processes in the accumulated organic top layer of the soil.[17a–d] Temperature sensitivity also indicates that chlorination in forest soils is a primarily biotic and enzymatic process. However, abiotic chlorination may also occur, in which iron(III) in soils plays a catalytic role.[18] Although key enzyme systems and relevant microorganisms are yet to be fully identified,[19a,b] it is very likely, as discussed herein, that fungal chloroperoxidases (CPOs) able to oxidize chloride to hypochlorous acid are involved [Eq. (1)].

$$H_2O_2 + H^+ + Cl^- \rightarrow HOCl + H_2O \quad (1)$$

Most researchers in the field use the monochlorodimedone assay (Figure 1) to detect and quantify CPO activity.

ε_{290nm} = 20.2 $mM^{-1}\cdot cm^{-1}$ ε_{290nm} = 0.2 $mM^{-1}\cdot cm^{-1}$

Figure 1. The monochlorodimedone assay is widely used to detect quantitatively halogenating activity. Monochlorodimedone is a 1,3-diketone with an activated carbon atom that is brominated or chlorinated by HOBr or HOCl to form the dihalogenated compound. The loss of absorbance of monochlorodimedone is recorded at $\lambda = 290$ nm.

Indeed, CPO activity has been observed in several soil extracts,[20,21] with properties similar to those of the CPO from *Caldariomyces fumago*.[22] A metagenomic-based survey on soil samples from a German forest showed that species of organisms involved in microbial (de)halogenation were present.[23] Genes were detected in the soil samples that corresponded to heme-dependent and vanadium haloperoxidases. However, the origin of these genes was not further specified. The objective of this review is to pinpoint which enzymatic systems may be involved in the natural formation of $CHCl_3$. The relevant properties of these systems, with respect to formation of $CHCl_3$, is discussed and, in particular, the potential role of vanadium CPOs is highlighted. With few exceptions, these vanadium CPOs have been largely ignored in the literature.[17b,19,24]

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2. Haloperoxidases and Halogenases

2.1. Non-Heme Bacterial Haloperoxidases and Flavin-Dependent Halogenases

Originally, bacterial perhydrolases found in bacteria such as *Pseudomonas fluorescence* were designated as CPOs. This bacterial species produces the chlorinated antifungal antibiotic pyrrolnitrin. These perhydrolases do not contain a heme group or metal ions.[25] Initially, it was assumed that such enzymes from these species were involved in substrate-/product-specific pathways. These and other bacterial non-heme non-metalloha-loperoxidases, in the presence of an organic acid and H_2O_2, catalyze unspecific halogenations by forming peroxyacid; a strong oxidizing agent that is able to directly oxidize halide ions to form hypohalous acid.[25] This reaction is, however, unlikely to occur under natural conditions because both a high concentration of the organic acid is required and millimolar-range concentrations of hydrogen peroxide. It is therefore important to note that not all bacterial non-heme CPOs are real haloperoxidases. In this case, the activity, perhydrolysis, is more a quirk of nature than a means to an end. Unfortunately, there are still examples in the literature that define these perhydrolases as CPOs. Unquestionably, there are a number of bacterial enzymes that are responsible for the formation of halogenated antibiotics, but these are typically dependent on flavin. These enzymes function through the intermediate formation of a flavin hydroperoxide formed by the reaction of oxygen, $FADH_2$ (FAD = flavin adenine dinucleotide), and a halide ion. The subsequently formed hypohalous acid diffuses along the protein's channel to form a long-lived chlorinated intermediate, which reacts with a specifically bound substrate.[26] HOCl does not leave the active site, which makes these substrate-specific, O_2-dependent halogenases unlikely to be involved in the formation of $CHCl_3$ in soils. In addition, the enzymatic activity of these flavin-dependent halogenases is very low (turnover number 1–7 min^{-1}).[27] This is in contrast to the study based on chlorine isotope effects by Aeppli et al.,[28] who suggested some involvement of these flavin-dependent halogenases in the formation of organochlorines.

2.2. Non-Heme Fe^{2+} Halogenases

This highly homologous group of chlorinases from, for example, *Streptomyces*, *Pseudomonas*, and *Lyngbya* species are involved in the biosynthesis of natural chlorinated products.[29a–e] They share about 20–57% sequence identity and use non-heme iron, O_2, and 2-ketoglutarate to catalyze the chlorination of α-amino acids at aliphatic side-chain positions. To function as a substrate, the amino acid must be linked as a thioester to a phosphopantetheine tether. The reaction mechanism involves a haloferryl species that cleaves aliphatic C–H bonds in the bound substrate, which results in the formation of halogenated intermediates. Recently, an iron-dependent halogenase was discovered in cyanobacteria that was able to chlorinate stereospecifically a free-standing Fischer indole.[29f] Again, this is a group of enzymes that has a very specific binding site for their substrates and it is not probable that other substrates are chlorinated.

Thus, free and diffusible HOCl is not formed and it is unlikely that these enzymes are a source of $CHCl_3$.

2.3. Heme-Containing Haloperoxidases

2.3.1. Heme-Containing CPOs

The classical representative of this class of enzymes is the heme-containing CPO from the filamentous fungus *C. fumago* discovered in 1959.[30] This fungus from the class of Ascomycetes produces caldariomycin; a cyclopentane derivative containing two chlorine atoms.[31] This enzyme belongs to the heme thiolate peroxidases and is well characterized.[32–34] It has been extensively studied for its synthetic applications because its peroxygenase activity allows hydroxylation of C–H bonds (benzylic hydroxylation), epoxidation of double bonds, and sulfoxidation. Although easily inactivated by the substrate, hydrogen peroxide, and its product, HOCl, the enzyme is popular for chlorination studies owing to its commercial availability and initial high turnover (1000 s^{-1}).[34] The low optimum pH (around 3.0) of the enzyme for chloride oxidation by hydrogen peroxide[34] displays a K_m for hydrogen peroxide of around 0.2 mM and for chloride of around 5 mM.[31,34] HOCl or HOBr formed has been postulated to be released and could thus nonspecifically halogenate organic compounds.[35] As shown in Figure 2, this CPO is readily inactivated above 50 °C. Also, storage at slightly elevated temperatures leads to rapid inactivation.[36]

Ron Wever is Professor in Biocatalysis at the University of Amsterdam, from which he retired in 2012. His research was focused on metalloproteins and their reactions with oxygen or oxygen derivatives. His group discovered a new class of enzymes, the vanadium haloperoxidases, in which vanadium is the prosthetic group. In 2016, he received the Vanadis Award for his ongoing contributions in elucidating the structure and function of these enzymes. His latest research activities also dealt with enzymes able to phosphorylate or sulfate compounds, with the aim of replacing chemical synthesis methods by more cleaner biocatalytic procedures.

Phil Barnett works as Assistant Professor in the Department of Anatomy, Embryology and Physiology at the Academic Medical Center in Amsterdam. He trained as a Ph.D. student in biochemistry under Ron Wever, focusing on protein structure and function of vanadium haloperoxidases (1997). After a post-doctoral position of three years studying peroxisomal protein import, he switched his research focus to another area of interest, namely, mammalian gene regulation and disease. His current research is set around trying to understand human genetic variation and its influence on transcriptional responses and conduction diseases of the heart.

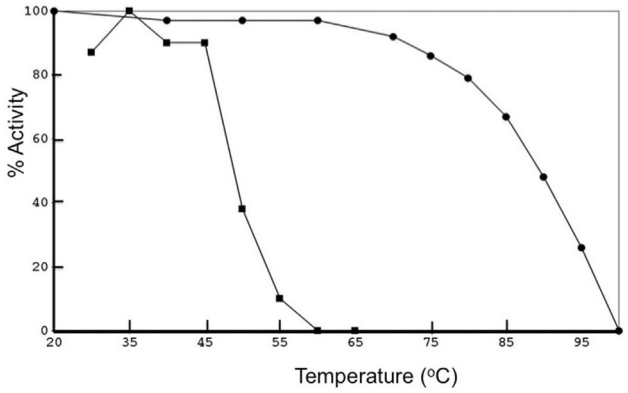

Figure 2. Stability of heme CPO (■) and vanadium CPO (●). The heme CPO was incubated for 15 min at the temperatures given and then diluted with buffer prior to analysis.[36] Samples of vanadium CPO were incubated for 5 min in Tris buffer (pH 8.3) at the given temperature, after which time the chlorinating activity was measured. Prolonged incubation (15 min) of the enzyme did not affect the midpoint temperature.[77] See Section 2.4.4. for details on the vanadium CPOs. The figure was constructed from the data given in refs. [36, 77].

Similar temperatures of inactivation of chlorination were also observed in other experiments observing weathering effects of plant material.[37]

2.3.2. Involvement of CPO in $CHCl_3$ Production by Soils

CPO activity has been detected in several spruce forest soil samples in crude extracts by using 0.2 M phosphate buffer, pH 3.6, containing 0.1 M KCl. Partial characterization of the detectable peroxidase showed activity only in the presence of hydrogen peroxide, and a pH optimum of around 3–3.5.[20,21] Further, storage at 23 °C resulted in complete inactivation within 4 days.[21] The pH optimum for chlorination by soil samples exposed to different pH values ranged from pH 2.5 to 4, with the highest activity observed in the surface organic layer.[21]

There is general consensus that a heme CPO is involved in the formation of organochlorines in soils, and consequently, in the formation of $CHCl_3$.[19a,b,38] That heme CPO is responsible for the formation of naturally produced organochlorines, and consequently, the formation of $CHCl_3$ has recently been challenged.[28] On basis of the difference in chlorine isotope effects of organochlorines produced by CPO in vitro and those found in soils, it was concluded that this enzyme barely had any role in the production of soil organochlorines or that there were other chlorination pathways. By using the same technique, it was shown,[39] however, that the value of the chlorine isotope effect of chloroform formed by the CPO-catalyzed chlorination of humic acid was strongly affected by the experimental conditions used, for example, pH and the concentrations of Cl^- and hydrogen peroxide. These findings provide further support to the role of CPO in organochlorine formation in soils. However, it is very unlikely that the CPO from the Ascomycete *C. fumago* is solely responsible for this. Organohalogen production is a ubiquitous capacity among commonly occurring Basidiomycetous fungi.[39] Several ecologically significant strains of Basidiomycetes were found to produce chlorinated anisyl derivatives when grown on natural lignocellulosic substrates. It can be hypothesized that the Basidiomycete *Mycena metata* and *Peniophora pseudopini* probably secrete CPOs based on the observation that these fungi produce $CHCl_3$ in the headspace of the pure cultures when grown on organic matter. However, these Basidiomycetes still only produce low levels of $CHCl_3$.[40] As discussed in Section 2.4, to this end, vanadium CPOs may also play a significant role in $CHCl_3$ production.

In situ X-ray spectroscopy studies showed the formation of stable chlorinated hydrocarbons in soils, sediments, natural waters, and weathering plant material.[41] Interestingly, these chlorinated organic molecules have been found in humic substances from various global sources; thus, their presence is independent of geographic location.[41] Organochlorines are rapidly formed in a process in which inorganic chloride (Cl^-) is incorporated into organic material, presumably through CPO activity. Surprisingly, these organochlorines are also found in degrading senescent leaves still attached to the tree, which suggests that organochlorine formation in weathering plants is due to the action of airborne, external, terrestrial organisms producing CPO activity during the invasion of weathering plants. As far as it is known, endogenous plant peroxidases have no CPO activity (Section 2.3.4). Several studies already showed that exposure of humic substances to the CPO from *C. fumago* resulted in the chlorination of aromatic groups and the formation of chlorinated volatile compounds and chlorinated acetic acids, of which the latter can decay to $CHCl_3$.[38,42,43] In a more recent K-edge X-ray study,[37] the formation of these organohalogens was studied by using the CPO from the fungus *C. fumago* with the needles of redwood (*Sequoia sempervirens*) as a substrate. The organochlorines formed in this study closely resembled those found naturally in weathering needles of redwood; this adds weight to previous studies.[41]

2.3.3. Related Fungal Heme Peroxidases

Distinct relatives of the heme CPO are aromatic peroxygenases (EC 1.11.2.1), also named unspecific oxygenases. They form a superfamily of heme thiolate peroxidases found in Basidiomycetes. The enzyme from the fungus *Agrocybe aegerita* has structural similarities to that of the heme CPO, but very different catalytic properties. It is able to incorporate an oxygen atom from peroxide into various aromatic, heterocyclic, and aliphatic compounds.[44a–c] In contrast to the heme CPO, this peroxygenase is only significantly able to oxidize bromide to HOBr, which leads to nonspecific bromination of compounds.[45] The chlorinating activity of this enzyme is very low,[44b] which is surprising because the high redox potential of the catalytic intermediate (compound I) of the peroxygenase[46] should facilitate oxidation of chloride to form HOCl. The related peroxygenase from the agaric fungus *Marasmius rotula* similarly displays almost no brominating activity,[47] which strongly suggests that these fungal heme thiolate peroxygenases have no capacity to form HOCl, leaving only the heme CPOs or other unidentified peroxidases as candidates involved in the formation of $CHCl_3$ in soils and forest litter.

2.3.4. Other Fungal Heme and Plant Peroxidases Possibly Involved in Halogenation

This group of peroxidases is subdivided into three classes on the basis of sequence similarity.[48] Class I corresponds to a group of intracellular enzymes, for example, ascorbate peroxidase from plants. Class II is the secretory fungal peroxidases, for example, those secreted by white-rot Basidiomycetes responsible for the degradation of lignocellulose. Under lignolytic conditions, the fungus *Phanerochaete chrysosporium* secretes two types of extracellular heme peroxidases: lignin peroxidase and manganese peroxidase. These enzymes have an important role in the breakdown of plant biomass, but are not able to oxidize chloride, although low brominating activity at pH 2 has been demonstrated.[49,50] Class III contains the secretory plant peroxidases, such as those from horseradish or soybean, of which the soybean peroxidase shows a level of brominating activity.[51] Interestingly, it was reported that an acid phosphatase from the embryonic axes of the plant *Phaseolus vulgaris* displayed CPO activity.[52a] However, this appears to be artificial CPO activity because the enzyme first had to be treated with ethylenediaminetetraacetic acid (EDTA) to remove metals and then the apoenzyme had to be incubated with vanadate. It was demonstrated earlier that the apo forms of bacterial acid phosphatases incubated with vanadate had haloperoxidase activity.[52b]

2.4. Vanadium Haloperoxidases in the Terrestrial Environment

Many of the vanadium iodo- and bromoperoxidases found in the marine environment (macroalgae and cyanobacteria) are involved in the production of large amounts of brominated and iodinated compounds that are released into the atmosphere.[1,2,53] Vanadium CPOs are also known and they are found in prokaryotes involved in the specific synthesis of chlorinated antibiotics and in a group of common terrestrial fungi, the dematiaceous Hyphomycetes, belonging to the phylum Ascomycota. A bromoperoxidase is also present in the lichen *Xantoria parietina*, which grows on rocks and stones.[54] As discussed below, these fungal CPOs are involved in the decay of lignocellulose and plant debris, and may also play important roles in the formation of chlorinated compounds derived from soil organic matter. Vanadium haloperoxidases differ significantly from the heme haloperoxidases.[55] They possess an oxidometalate (vanadate) as a prosthetic group, which makes them very resistant towards oxidative inactivation. These enzymes, unlike classical heme peroxidases, are only able to oxidize halides in the presence of hydrogen peroxide to form hypohalous acids with pH optima tending towards physiological ranges found in soils.

2.4.1. Bacterial Vanadium-Dependent CPOs

Recently, vanadium CPO-encoding genes were identified in a napyradiomycin biosynthetic gene cluster from marine sediment-derived Actinomycete *Streptomyces* sp. CNQ-525.[56a,b] The CPO involved has a very high selectivity and affinity for its substrate, a meroterpenoid, which is an intermediate in the synthesis of napyradiomycin antibiotics, and appears unable to form HOCl or chlorinate other compounds. Similarly, a vanadium-dependent CPO from the marine *Streptomyces* sp. CHN-189 was shown to catalyze the biosynthesis of the merochlorin meroterpenoid.[57a,b] This enzyme catalyzes site-selective naphthol chlorination of the substrate pre-merochlorin and appears unable to chlorinate other substrate analogues or to form HOCl. These results suggest that terrestrial *Streptomyces* species only specifically chlorinate antibiotics and are not a source of $CHCl_3$ found in soils.

2.4.2. Occurrence of Other Fungal Species with Haloperoxidase Activity

About 30 years ago, a research group at the Cetus Cooperation made the important discovery that several pathogenic fungal species in the class of dematiaceous Hyphomycetes showed haloperoxidase activity.[58] This class belongs to the Deuteromycetes of fungi imperfecti, which is a provisional taxonomic group for which no sexual stage has been observed. Most Hyphomycetes are now assigned to Ascomycetes on the basis of DNA analysis. They reproduce by sporulation in a process called sporogenesis. These fungal species were originally isolated from soil samples, plants, and plant debris in Death Valley. This ecosystem was chosen on purpose because organisms growing in this habitat should be resistant towards extreme conditions and the enzymes present in these microorganisms may be more thermostable and show more alkaline pH optima, as required in some industrial applications. By using an assay based on the bromination of phenol red to bromophenol blue (Figure 3), several species (*Cladosprium* sp., *Embellisia alli*, *Ulocladium chartarum*, *Embellisia* sp., and *Drechslera halodes*) were shown to possess bromoperoxidase activity with pH optima above 3.0.[58]

Shortly after, it was demonstrated that haloperoxidases isolated from the fungal species *Curvularia inaequalis* and *Drechslera* sp. were able to produce HOCl at pH 5. These enzymes also demonstrated resistance towards high hydrogen peroxide concentrations and HOCl, unlike heme-containing haloperoxidases.[59] Interestingly, these CPOs were devoid of a heme group.[60] A more detailed study by using the monochlorodimedone assay (Figure 1) showed that the genera *Alternaria*, *Curvularia*, *Drechslera*, *Ulocladium*, and *Botrytis* also produced

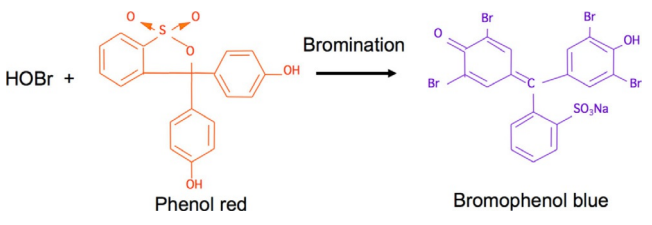

Figure 3. Bromination of phenol red used to detect halogenating activity qualitatively. Owing to bromination, a marked color change occurs from yellow (at acidic to neutral pH) to deep blue or purple, which can easily be detected by eye.

high levels of CPO activity.[61] Specifically, *Alternaria alternata*; *Curvularia cymbopogonis*, *C. inaequalis*, *C. tuberculata*, *C. verruciformis*, and *C. lunata; Dichotomophthora portulacae*; *D. halodes* and sp.; *Ulocladium* sp.; *Botrytis cinerea* and sp.; *Humicola fuscoatra*; and *E. alli.* Some of these fungi were obtained from grapes (*Botrytis*), others from soil samples (*Drechslera* sp. from British Virgin Islands and New Jersey; *Ulocladium* sp. from South America and Death Valley). Hunter et al. also showed that moderate CPO levels were produced by *Dendryphiella salina*, *Dendryphion nanum*, *U. chartarum*, *Bispora betulina*, *Stemphylium macrosporidium*, *Wardomyces anomalus*, and *Cladosporium* sp.[61]

2.4.3. Fungal Vanadium CPOs in the Terrestrial Environment

Van Schijndel et al. established that the CPO from *C. inaequalis* contained vanadium as a prosthetic group,[62] and that CPOs from *Ulocaldium chartarum*, *Embellisia didymospora*, *Drechslera subpapendorfii*, and *B. cinerea* were also vanadium enzymes.[63–65]

From the data, it is fair to conclude that most, if not all, of the haloperoxidases found in the family genera of dematiaceous Hyphomycetes produce CPO activity. Indeed, the peroxidases database[66] and our own BLAST search with the mRNA sequence of the vanadium CPO from *C. inaequalis* as a query (Figure 4) show that in the phylum Ascomycota many pathogenic fungal species are present that have mRNA sequences corresponding to the known sequence of vanadium from CPOs, for example, the rice blast fungus *Magnaporte grisea*, the fungal pathogen of wheat *Phaeosphaeria nodorum*, and the necrotrophic plant pathogen *Pyrenophora tritici-repentis*.

These Hyphomycetes are very common and usually saprophytic in nature, and can be found on living plants or nonliving organic matter. They constitute the predominant flora in terrestrial habitats and are isolated readily from leaf litter and differing soils types, for example, soils from the coastal areas of India,[67] salt marshes in Egypt,[68] and soils from Death Valley.[58] Furthermore, they may be present as pathogens that cause diseases in different host plants, typically agricultural crops,[69] and thus, represent a potential threat to food production. Examples include *Alternaria*, which causes a destructive foliar disease in potato and tomato plants,[69] and *Curvularia* species, which are facultative pathogens of plants and grasses.[69,70] Most of these deuteromycetes rely upon conidia for reproduction spores that become airborne. In fact, *Botrytis*, *Cladosporium*, *Alternaria*, and *Curvularia* species are almost always present in air samples[71] and spores can be isolated from plant surfaces. Plants and leaf matter normally undergo seasonal decline to become moribund. It has been demonstrated that parasitic forms of the fungi can infect living growing plants when spores land and germinate on a host plant.[72,73]

2.4.4. Properties of Vanadium CPOs

Many structural and kinetic data are available for these enzymes.[55,74–76]

Figure 5 shows the structure of the active site and the catalytic mechanism of these enzymes.

Compared with heme CPOs, these enzymes are highly stable and possess characteristics similar to those of enzymes from thermophiles. Their midpoint temperature is unusually high, as

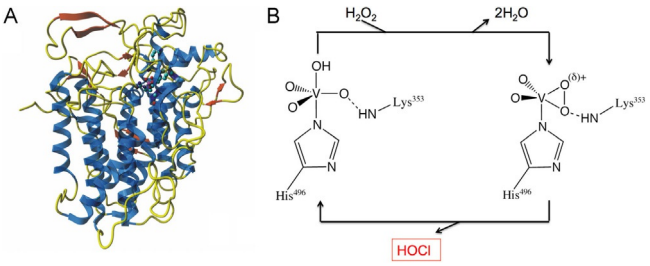

Figure 5. A) X ray structure (PDB structure 1VCN) of vanadium CPO from *C. inaequalis*.[74] B) The catalytic mechanism of vanadium haloperoxidases. Vanadate in the enzyme first reacts with hydrogen peroxide to form a peroxido intermediate, which becomes protonated. Hydrogen-bonded lysine 352 activates the bound peroxide. In the next step, a chloride ion reacts with the activated peroxide and HOCl is produced, which leaves the active site.[77]

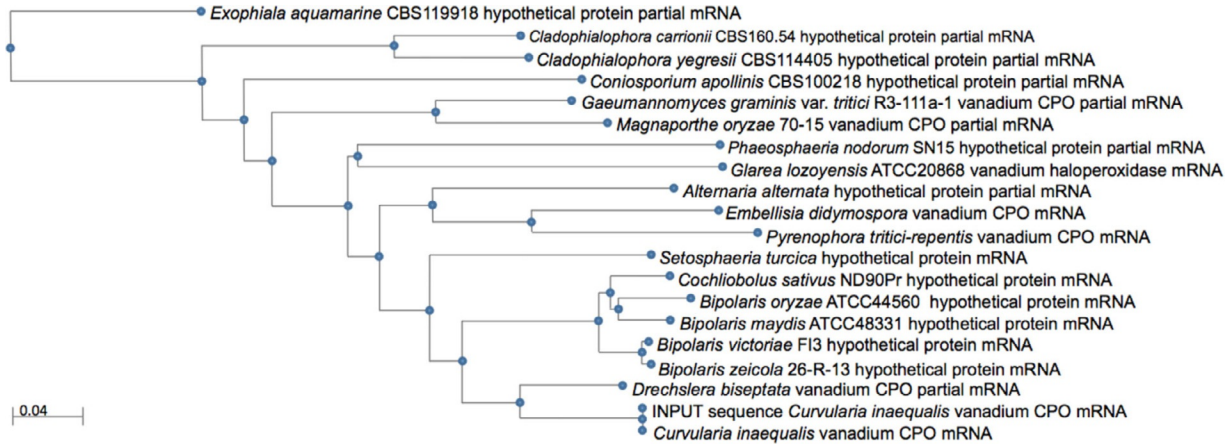

Figure 4. The BLAST tree output after performing a BLAST search with the mRNA of the vanadium CPO (ENA X85369.1) from *C. inaequalis* as an input sequence. Parameters used were default using optimized for "somewhat similar sequences" within the fungi taxids: 4751 and 33154. BLAST hits with $p < 3 \times 10^{-15}$ were selected for tree building by using fast minimum evolution.

illustrated in Figure 2. The midpoint temperatures of the CPOs from *Curvularia inaeqalis*[77] and *Drechslera biseptata*[63] are about 90 and 82 °C, respectively, and that of the related CPO from *Embellisia didimospora*[64] is about 65 °C. The thermostability of these enzymes is thus much higher than that of the heme CPO, which inactivates above 47 °C. The pH optimum of chlorinating activity is approximately pH 5.5, with an activity range from pH 4.5 to 7.[62,63,77] This pH optimum is higher than that of the heme enzyme, which is pH 3,[34] although the turnover of about 20 s^{-1} is considerably lower than that of the heme CPO. The kinetic parameters for the vanadium CPO are noteworthy. In particular, the K_m value for hydrogen peroxide of 5 μM at pH > 5 (Figure 6) is unusually small.[62,63] This means that the enzyme is functional at very low peroxide concentrations. Similarly, the K_m value for chloride at pH 5 is 0.5 mM, which increases linearly with pH (Figure 6).

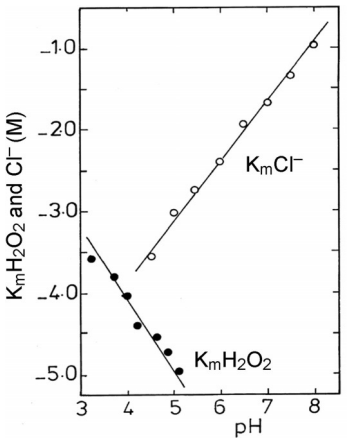

Figure 6. Log K_m values for chloride and hydrogen peroxide as a function of pH.[55] Reproduced with permission from Springer.

These values are smaller than those reported for the heme CPO.[33] The pH optimum and the kinetic parameters show that these vanadium enzymes would certainly be active in soils or environments with neutral to slightly alkaline pH values. A study of topsoil pH of arctic and subarctic sites and ecosystems showed that there were large variations in pH between locations and within sampling sites.[14] In northern Sweden (subarctic forest soil), the mean pH value varies between 4.2 and 4.7. In Greenland, arctic and subarctic mean pH values are found between 5.6 and 6.7. Similarly, Redon et al. reported pH values in grassland water and arable land varying from 5.0 to 8.3, whereas those found in forests were typically lower.[12] Most lakes in Australia are pH-neutral to slightly alkaline[15] and sediments have been shown to release organochlorines, including $CHCl_3$, in a biotic process. Many of these reported pH values are far above the optimum pH activity ranges of heme CPOs,[34] but certainly accommodate the activity range of vanadium CPOs.

In line with thermal stability, these enzymes are also resistant towards denaturation. In fact, they remain active in solutions containing 1% sodium dodecyl sulfate[62] and only become inactivated when these solutions are boiled. Similarly, they remain active when stored in organic solvents (solutions of 40% methanol, ethanol, or 2-propanol) for 3 weeks,[77] and are very resistant towards oxidative inactivation by hydrogen peroxide, singlet oxygen, or HOCl.[78,79] However, these enzymes are rapidly inactivated by phosphate, in particular, under mildly acidic pH values. This is probably due to an exchange of vanadate in the active site for its structural analogue, phosphate. Therefore, in studies of these enzymes or in extraction of peroxidase activity from soil samples, the use of phosphate buffers should be avoided. The presence of low concentrations of peroxide completely prevented this inhibition.[80] Because binding of vanadate requires no additional enzymes or modifications to amino acid or metal groups, reconstitution of activity of the apoenzyme can be simply carried out through the addition of 10 μM vanadate. This concentration may seem high, but it is well documented that soils contain 100 ppm vanadium, which would correspond to approximately 2 mM.[81] This is already sufficiently high to obtain a fully reconstituted enzyme in the in vivo situation. More importantly, the presence of hydrogen peroxide, even at low concentrations, increases the affinity for vanadate considerably. This is due to the formation of pervanadate, which has a much higher affinity (< 5 nM) for the enzyme than that of vanadate itself.[82]

3. Biological Function of Fungal Vanadium CPOs

After germination of the conidia on or in their host, the fungal hyphae extend continuously at their extreme tips, from which enzymes are released into the environment to degrade the substrate polymers and allow penetration of the underlying surface (Figure 7). See ref. [83], for further details on how

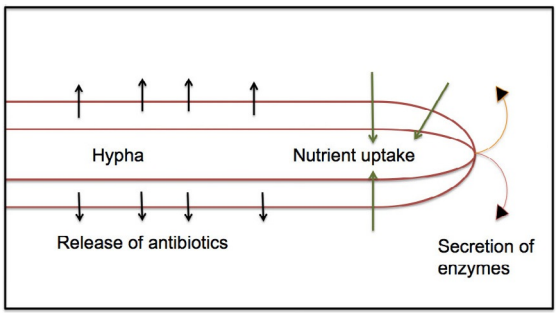

Figure 7. The tip of a growing hypha, which release enzymes and antibiotics, and takes up nutrients from the environment.

pathogenic plant fungi penetrate plant leaves.

From the tip extension, antibiotics or other inhibitors are released subapically into the substrate erosion zone to prevent competing organisms from using the enzyme digestion products. Experiments by Barnett and co-workers illustrated that CPO was also excreted by the growing hyphae of *Curvularia inequalis* (Figure 8).[84,85] Spores were inoculated on a nitrocellulose filter placed on top of nutrient agar, with a second embedded nitrocellulose filter on the bottom, and the fungal my-

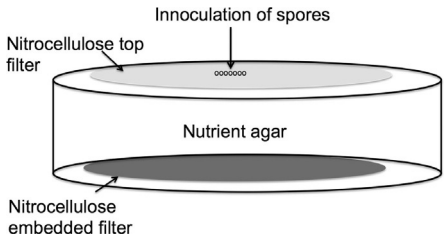

Figure 8. The agar plate system used to demonstrate the secretion of the vanadium CPO from the living, extending fungal tip.

celium was allowed to grow for 5 and 7 days, respectively.[85] After 5 days of incubation, CPO activity was only observed on the top filter. After 7 days, when the tips of the growing hyphen reached the embedded filter, CPO activity was also found on the embedded filter. This shows that the enzyme is secreted into the medium and found on the surface of the growing hyphal tips. This localization is substantiated by the finding that, after growing the fungus, treatment of the fungal mass with a low concentration of a detergent (0.05% sodium dodecylsulfate) results in extraction of active CPO activity. The amount of CPO secreted by the growing fungus is substantial and constitutes approximately 60–70% of total isolatable protein.[84] In glucose-rich environments, when the enzyme is not needed, the transcription of vanadium CPO is toned down.[84]

It is known that HOCl itself is a strong oxidizing agent able to disrupt and oxidize a diversity of structures. These observations have led to the proposal that the function of the CPO is to oxidize the waxy protective cuticle layer on the leaves of the plant and degrade the cell walls of the host (plants) to facilitate penetration of the fungus in the leaf cuticle and plant cell to reach nutrients in the cell.[84,85] HOCl itself may have this role directly, but it is well documented that stressing plants, for example, by high-intensity light, damage, or pathogen attack, leads not only to the formation of hydrogen peroxide, but also to superoxide radicals.[86] HOCl may react with O_2^- or H_2O_2, according to Equations (2) and (3), respectively.[87]

$$HOCl + O_2^- \rightarrow OH^{\bullet} + Cl^- + O_2 \quad (2)$$

$$HOCl + H_2O_2 \rightarrow {}^1O_2 + Cl^- + H^+ + H_20 \quad (3)$$

The hydroxyl radicals formed in Equation (2), together with singlet oxygen formed in Equation (3), may be more effective than HOCl alone. Figure 9 illustrates this proposal, as postulated by Barnett.[85]

It is expected that HOCl generated by these fungal species in the early stages of infection of plants or leaves will result in the formation of chlorinated intermediates. As illustrated by Leri and Myneni,[88] degradation of plant matter will probably

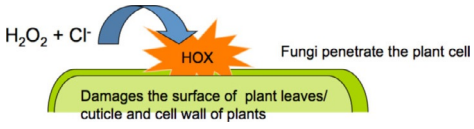

Figure 9. Oxidation and damage of the cuticle and epidermal cell structures by HOCl to allow penetration of the fungal hyphal tip into the plant host.

continue, even after plant death, given the extreme stability of these vanadium CPOs. They showed that aromatic organochlorine species were detectable only in leachates from the first month of degradation, probably originating from senescent leaves in the forest ecosystem, consisting of oak, maple, and pine trees. Interestingly, these aromatic chlorine species are also produced in decaying plant litter by a process that depends on oxidized chlorine species produced by fungal CPOs. Aliphatic organochlorine species have also been observed in healthy leaves that then persist throughout senescence and humification and contribute to the pool of organochlorines in the soil.[88] These are superficial compounds that may originate from the cuticle, which forms a protective layer on the leaf surface. It is therefore tempting to speculate that these originate from early stages of infection by dematiaceous fungi. In line with this, studies making use of X-ray microscopy, combined with optical spectroscopy, showed that fungi played a role in the production of aromatic organochlorine species from natural organochlorine formation in weathering plants.[89] In fact, inoculation of healthy detached oak leaves with the pathogenic fungi *Fusarium oxysporum* resulted in the formation of aromatic organochlorine species, as evidenced by Cl 1s X-ray absorption near-edge structure spectra. Unfortunately, the presence of CPO in *Fusarium* has not yet been shown. Only one research group has elaborated on the possible role of dematiaceous Hyphomycetes in this process and the potential role of the vanadium enzymes. Direct formation of halogenated metabolites by the plant pathogenic fungus *C. inaeqalis* has indeed been reported,[24] and it was shown that *C. inaeqalis* generated chlorinated aromates from lignin during its degradation of aspen wood, resulting in the production of 2-chlorolignine fragments (5-chlorovanillin and 2-chlorosyringaldehyde). Metagenomic analysis of forest soil samples also shows the detectable presence of genes corresponding to vanadium haloperoxidases.[23] Unfortunately, the source of these genes is not clear, although based on fungal presence, one would certainly expect the presence of vanadium CPOs. Figure 10 gives an overall picture of the chemical processes that occur after chlorination of organic matter by CPOs. In the reaction shown in Figure 10, a resorcinolic structure, which is a common structural element in humic material, is chlorinated. After chlorination of the aromatic ring, hydrolysis occurs and the formed product is further chlorinated and decays at pH values above 7 to form $CHCl_3$, whereas trichloroacetic acid is produced at more acidic pH values.[8a,b] The formed $CHCl_3$ is ventilated to the atmosphere and in the stratosphere chlorine radicals are formed through UV light-induced photolysis. This will result in ozone depletion by well-known reaction paths.

4. Summary and Outlook

In forest soils, heme CPOs are present that produce HOCl, which leads indirectly, through the chlorination of natural organic matter, to the formation of $CHCl_3$. These forest soils are, in general, acidic in nature, fitting pH optima and other properties of the enzyme activity isolated from soil samples. To date, the only heme CPO to have been well characterized is

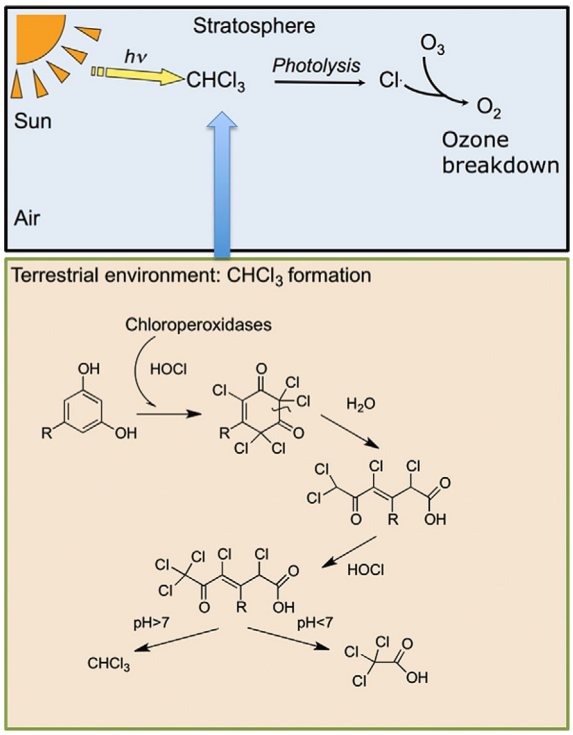

Figure 10. Formation of $CHCl_3$ in the terrestrial environment and its fate in the stratosphere, where chlorine atoms become involved in catalytic ozone breakdown.

that of the sooty mold *C. fumago*. This heme CPO has served as the model enzyme for several in-depth chlorination studies of plant matter and other organic matter naturally present in soils. The sooty molds are a cluster of species[90] that live on plant surfaces and unlike the Basidiomycetes they are not sustained in soils, but typically grow on fruits and leaves. Therefore, the role of Basidiomycetes in chlorination reactions in soils is not clear. However, considering the fact that these Basidiomycetes produce large amounts of organochlorines, heme CPOs should be present.

Many of the mostly pathogenic fungi that secrete CPO activity are ubiquitously present in nature and, considering the special properties of the vanadium CPO, these enzymes will almost certainly play an important role in the formation of $CHCl_3$. This occurs, in particular, in unforested areas, such as marshes, tundras, lakes, grassland, and agricultural soils, which, in general, have a more neutral to slightly alkaline pH value. The contribution of Hyphomycetes to the formation of $CHCl_3$ in soils has been largely ignored. Furthermore, considering the unusual stability and their presumed longevity, these vanadium enzymes once formed inside or on the surface of the plant host will probably be found associated with degrading plant matter and in soils. Studies to establish their presence and diversity in soils are essential. Another question that should be addressed is will the vanadium CPO survive in soils owing to the presence of phosphate? Soils indeed contain soluble orthophosphate in amounts that vary with the type of soil and pH, and the presence of orthophosphate may lead to inactivation of the VCPO. However, very low concentrations of hydrogen peroxide prevent inactivation by phosphate.[80] Field experiments will give an answer.

$CHCl_3$ formed by the fungi is subsequently released into the atmosphere and, as a result of photolysis in the stratosphere, chlorine radicals will be formed that cause depletion of ozone. As such, fungi contribute to the global chlorine cycle, in which CPOs play a primary role in the formation of $CHCl_3$, and thus, in the natural breakdown process of the earth's ozone layer. Natural breakdown of ozone also occurs owing to the activity of vanadium bromoperoxidases present in seaweed, phytoplankton, and cyanobacteria. These organisms produce huge amounts of bromoform and other volatile brominated compounds that are ventilated to the atmosphere.[2a,91]

Conflict of interest

The authors declare no conflict of interest.

Keywords: atmospheric chemistry · environmental chemistry · metalloenzymes · pathogenic fungi · vanadium

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