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Hypothiocyanous Acid: An Overview

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Abbreviations

EPO	eosinophil peroxidase
HOSCN	hypothiocyanous acid
HO_2SCN	cyanosulfurous acid
LPO	lactoperoxidase
Nbs	5-thio-2-nitrobenzoic acid
$NCS\text{-}O\text{-}SCN^{2-}$	dithiocyanate ether anion
OCN^-	cyanate
$OSCN^-$	hypothiocyanite
SCN^-	thiocyanate
$(SCN)_2$	thiocyanogen
$S(CN)_2$	sulfur dicyanide
$(SCN)_3^-$	trithiocyanate
$SCNO^-$	isomeric triooxime

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Abstract

Two important peroxidases, eosinophil peroxidase (EPO) and lactoperoxidase (LPO), catalyze the oxidation of thiocyanate to yield products such as hypothiocyanous acid and hypothiocyanite. These products have been shown to exhibit antimicrobial action, suggesting that these systems serve an important role in innate immunity. However, the underlying mechanisms of thiocyanate oxidation have remained elusive. This review will address the chemistry, biochemistry, and detection methods of hypothiocyanous acid (HOSCN) and its conjugate base hypothiocyanite ($OSCN^-$).

Introduction

Peroxidase-catalyzed oxidation of halides (Cl^-, Br^-, I^-) and pseudohalides (SCN^-) by H_2O_2 yield potent oxidizing agents that exhibit antimicrobial activity *in vivo*, presumably by damaging vital structural and functional microbial components. Although various peroxidases have been shown to oxidize thiocyanate (SCN^-) *in vitro*, only lactoperoxidase (LPO) and eosinophil peroxidase (EPO) are believed to utilize thiocyanate to generate antimicrobial oxidizing agents *in vivo*. The predominant oxidizing agents produced are hypothiocyanous acid (HOSCN) and its conjugate base hypothiocyanite ($OSCN^-$).

The lactoperoxidase/H_2O_2/ SCN^- system is present in saliva, breast milk, and tears where it is thought to play an important role in innate immunity [1]. Recently, the LPO system was shown to be present in airway mucosa where it is hypothesized to exhibit host defense properties and possibly play a role in airway diseases such as cystic fibrosis [2].

Eosinophils have been well characterized in their role to mediate the extracellular destruction of helminthes and other metazoan pathogens [3]. Eosinophils are also recruited to the airways in asthmatic patients and other allergic inflammatory settings, whereby they contribute to host tissue damage. Further, eosinophils accumulate at the sites of various cancers. EPO is present in abundant amounts in granules of eosinophils [4], and EPO inhibitors nearly abrogate the ability of eosinophils to kill extracellular parasites [5]. Since thiocyanate is a primary substrate oxidized by LPO/EPO and H_2O_2, characterization of the oxidants produced is

necessary to fully understand the contribution of LPO and EPO in both physiological and pathological settings aforementioned.

Chemistry of Hypothiocyanous Acid

Early studies by Wilson and Harris determined that hypothiocyanite ($OSCN^-$) is formed as a stable intermediate in the oxidation of thiocyanate (SCN^-) by hydrogen peroxide (equation 1) [6]. Equation 1 proceeds through a simple two-electron reduction of H_2O_2 to $OSCN^-$ and H_2O, versus through intermediate Fenton-like radicals produced by one-electron reductions [7]. The production of $OSCN^-$ can also occur through the hydrolysis of thiocyanogen ($(SCN)_2$) (equation 2) [6].

$$SCN^- + H_2O_2 \rightarrow OSCN^- + H_2O \quad \{\text{equation 1}\}$$

$$(SCN)_2 + H_2O \leftrightarrow OSCN^- + SCN^- \quad \{\text{equation 2}\}$$

With a pK_a of 5.3, the percentage of HOSCN/$OSCN^-$ in the form of HOSCN is 67% at pH 5, 17% at pH 6, and 2% at pH 7 [6]. This is of biological importance because the pH in the oral cavity is often <5, which suggests that HOSCN is the predominant species. Since HOSCN is uncharged, it should freely cross the microbial membrane to oxidize with critical intracellular proteins. The partition coefficient in 2-octanol is 2.3 for HOSCN which suggests good lipid/membrane solubility [8].

Once formed, HOSCN/$OSCN^-$ undergoes a number of reactions to yield end products that differ depending on the pH state. Under acidic conditions (pH < 2), HOSCN is further oxidized in an acid-catalyzed fashion to yield end products cyanide (HCN) and sulphate (equation 9). Wilson and Harris proposed that in the excess of H_2O_2, HOSCN is oxidized to cyanosulfurous acid (HO_2SCN) (equation 4). Cyanosulfurous acid could further react to yield

HCN (equation 5). Cyanide could then react with HOSCN to form sulfur dicyanide ($S(CN)_2$) (equation 6). The rate law was determined to be third order (equation 10) [6].

$$H^+ + SCN^- + H_2O_2 \rightarrow HOSCN + H_2O \quad \{k = 3.3 \times 10^{-2}\ M^{-2}\ s^{-1}\} \quad \{\text{equation 3}\}$$

$$HOSCN + H_2O_2 \rightarrow HO_2SCN + H_2O \quad \{\text{equation 4}\}$$

$$HO_2SCN + 2H^+ \rightarrow H_2SO_4 + HCN \quad \{\text{equation 5}\}$$

$$HOSCN + HCN \rightarrow S(CN)_2 + H_2O \quad \{\text{equation 6}\}$$

$$HOCN + 2\ H_2O \rightarrow NH_4^+ + HCO_3^- \quad \{\text{equation 7}\}$$

$$H_2SO_3 + H_2O_2 \rightarrow H_2SO_4 + H_2O \quad \{\text{equation 8}\}$$

Overall stiochiometry for this acid catalyzed reaction obeys the following equation:

$$3\ H_2O_2 + SCN^- + \rightarrow HSO_4^- + HCN + 2\ H_2O \quad \{\text{equation 9}\}$$

$$R = k[H^+][\ SCN^-][H_2O_2]^2 / \{[H_2O_2] + \alpha[HCN]\} \quad \{\text{equation 10}\}$$

However, the mechanisms proposed by Wilson and Harris have been challenged by data recently published by Figlar and Stanbury [7, 9]. They propose that the reaction between H_2O_2 and SCN^- would yield thiocyanogen ($(SCN)_2$) and trithiocyanate ($(SCN)_3^-$) as intermediates under conditions of high H^+ and SCN^-. Moreover, they suggest that $(SCN)_2$ and $(SCN)_3^-$ are in equilibrium with HOSCN which is central to the mechanism (equations 11-12).

$$(SCN)_2 + SCN^- \leftrightarrow (SCN)_3^- \quad \{\text{equation 11}\}$$

$$(SCN)_2 + H_2O \leftrightarrow HOSCN + SCN^- + H^+ \quad \{\text{equation 12}\}$$

Under less acidic pHs (range 4<pH<12), hypothiocyanite decomposes to yield sulfate (SO_4^{2-}), ammonia (NH_4^+), and bicarbonate (HCO_3^-) (equations 13-16) [6,11].

$$OSCN^- + H_2O_2 \rightarrow OS(O)CN^- + H_2O \quad \{\text{equation 13}\}$$

$$OS(O)CN^- + H_2O_2 \rightarrow H_2SO_3 + OCN^- \quad \{\text{equation 14}\}$$

$$SO_3^{2-} + H_2O_2 \rightarrow SO_4^{2-} + H_2O \quad \{\text{equation 15}\}$$

$$HOCN + 2\ H_2O \rightarrow NH_4^+ + HCO_3^- \quad \{equation\ 16\}$$

Overall stiochiometry for this uncatalyzed reaction obeys the following equations:

$$4\ H_2O_2 + SCN^- + \rightarrow HSO_4^- + HOCN + 3\ H_2O \quad \{equation\ 17\}$$

$$HOCN + 2\ H_2O \rightarrow NH_4^+ + HCO_3^- \quad \{equation\ 18\}$$

This oxidation reaction was determined to be second order: first order with respect to both SCN^- and H_2O_2 or rate = k $[SCN^-]$ $[H_2O_2]$ [6]. However, a recent study by Egeberg *et al*., has questioned this rate law [11]. Employing capillary electrophoresis, their results suggest that the rate law for the oxidation reaction is first order with respect to H_2O_2 and zero order with respect to SCN^-.

Detection Methods

Measurement of $HOSCN/OSCN^-$ is commonly carried out using the 5-thio-2-nitrobenzoic acid (Nbs) assay. Oxidation of Nbs, which absorbs light at 412 nm, by 1 mol of $HOSCN/OSCN^-$ converts 2 mol Nbs into a colorless disulfide ((5,5-dithiobis(2-nitrobenzoic acid)). The concentration of the oxidizing agent is calculated from one-half the difference between the amount of Nbs added and the amount remaining (equation 19).

$$[HOSCN/OSCN^-] = ½\ (\text{Amount of Nbs added} - \text{amount of Nbs remaining}) \quad \{equation\ 19\}$$

To determine the products of peroxidase/SCN^-/H_2O_2 systems, one could use NMR analysis in conjunction with electrospray ionization mass spectroscopy (ESI-MS) [12]. Using $[^{13}C]$ SCN^- substrate, NMR analysis allows enumeration of the major products (Fig. 1). However, NMR does not identify the structures of the products. The use of ESI-MS reveals the structures of the

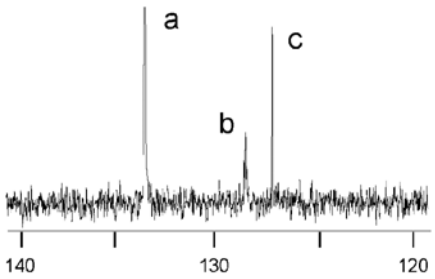
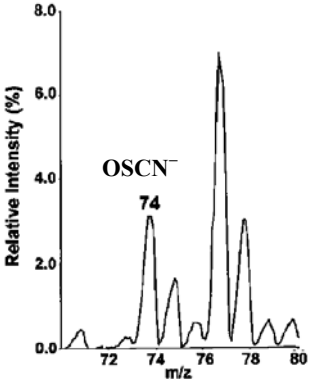


Fig. 1. **NMR analysis of EPO/SCN^-/ H_2O_2 system reaction products.** Peak *a* represents parent SCN^- resonance, whereas peaks *b* and *c* represent products



with *m/z* 74 peak is compatible with $OSCN^-$.

products (Fig. 2). Recently, capillary electrophoresis was used to measure concentrations of reaction products. Briefly, identities of products are determined by comparing the migration times of the anions separated during the reaction to migration times of standards [11].

Biochemistry of Hypothiocyanous Acid & Hypothiocyanite

Thiocyanate is generated *in vivo* as a product of cyanide detoxification [13], and is present at levels of 20-120 μM in serum and extracellular fluid [14] and 800 μM – 6 mM in saliva with the wide range attributed to smoking [15]. At these physiological concentrations, thiocyanate is the predominant substrate oxidized by lactoperoxidase (LPO) [6] and possibly eosinophil peroxidase (EPO) [15].

Similar to the non-enzymatic mechanisms, the predominant oxidative products produced by EPO and LPO are highly debated. The majority of the data suggests that $HOSCN/OSCN^-$ and HOCN are the major products of SCN^- oxidation (equations 20-22).

$$H^+ + SCN^- + H_2O_2 \xrightarrow{LPO\ /\ EPO} HOSCN + H_2O \qquad \{equation\ 20\}$$

$$HOSCN + H_2O_2 \rightarrow HO_2SCN + H_2O \qquad \{equation\ 21\}$$

$$HO_2SCN + H_2O_2 \rightarrow H_2SO_3 + HOCN \qquad \{equation\ 22\}$$

This mechanism is consistent with SCN^- acting as a pseudohalide, in that hypochlorous acid (HOCl) is the major product generated by myeloperoxidase (MPO)-mediated oxidation of Cl^-. However, other groups have proposed alternative initial products, such as dicyanate ether (NCS-O- SCN^-) [16], $^-OSC^{\bullet}N^-$ [17], and CN^- [18].

The $LPO/SCN^-/H_2O_2$ and $EPO/SCN^-/H_2O_2$ systems have been well characterized in terms of antimicrobial activity. Hypothiocyanous acid and accompanying oxidants have been shown to comprise a potent sulfhydryl-targeted cytotoxic system. In solutions containing

HOSCN/$OSCN^-$, essential sulfhydryl moieties of microbial proteins are oxidized to alkylsulfenyl thiocyanates (R-SSCN) [1] sulfenic acids (RSOH), and N-thiocarbamates ($RSC{=}ONH_2$) [19].

The potency and specificity of this SH-targeted cytotoxic system was demonstrated by Arlanson *et al.* [12]. Red blood cells (RBCs) challenged with $OSCN^-/OCN^-$ were susceptible to SH-oxidation-mediated damage. Intracellular stores of glutathione were first depleted, followed by glutathione-S-transferase and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and then membrane ATPases which culminated with cell lysis. Further, they showed that HOSCN inactivated RBC enzymes 10-100x more potently than other hypohalous acids (HOCl, HOBr). This SH-targeted cytotoxic system is thought to account for the antimicrobial activity in both the LPO- and EPO-/SCN^-/H_2O_2 systems. This system markedly differs from MPO/ H_2O_2/HOCl system which reacts rapidly with a variety of integral membrane constituents of phagocytosed pathogens, with minimal oxidation of intracellular moieties.

Although mammalian cells are killed by EPO- and LPO-derived oxidants, 10-fold higher concentrations of oxidant (ED_{50} = 200-300 uM) are required for killing as compared to schistosomules (ED_{50} = 20 uM) [12] and *Streptococcus viridians* (ED_{50} = 4-20 uM) [20]. To account for this selective antimicrobial killing, Ashby *et al*., hypothesize that the SH-targeted cytotoxic system is selective against prokaryotes because vital microbial structures are contained within the periplasm. The periplasm lacks reducing agents, namely glutathione, which serve a protective role in eukaryotic cells [19].

Aside from antimicrobial activity, MPO/H_2O_2/SCN^- system-derived oxidants have recently been shown to catalyze lipid oxidation of LDL [21]. Since SCN^- levels are elevated in smokers [15], the MPO/H_2O_2/SCN^- system may play a role in LDL oxidation and the pathogenesis of atherosclerosis.

Summary and Conclusions

In summary, the oxidation of SCN^- yields interesting chemistry and biochemistry that is a topic of much debate. The data strongly suggest that HOSCN/ $OSCN^-$ are the major products of both non-enzymatic and peroxidase-mediated oxidation of SCN^-. Most importantly, oxidation of SCN^- produces oxidants that comprise a potent SH-targeted antimicrobial system. This system has been shown to effectively kill extracellular parasites, suggesting a niche for the EPO/H_2O_2/SCN^- system in the eosinophil arsenal. Further, oxidants derived from the LPO/H_2O_2/SCN^- system are bactericidal suggesting a potential role in innate immunity, as LPO is stationed at sites where the host interacts with the external environment (e.g., oral cavity, airway mucosa). Of clinical importance, understanding the mechanisms underlying SCN^- oxidation may unveil important information in the pathogenesis of chronic airway infections, asthma, and others disease settings.

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