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# Lactoperoxidase

by

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## Abbreviations

**DBNBS-** 3,5-Dibromo-4-nitrosobenzenesulfonic acid
**DMPO-** 5,5-Dimethyl-1-pyrroline N-oxide
**EPO-** Eosinophil peroxidase
**EPR-** Electron paramagnetic resonance spectroscopy
**LPO-** Lactoperoxidase
**MALDI-MS-** Matrix assisted laser desorption ionization mass spectrometry
**MPO-** Myeloperoxidase
**NMR-** Nuclear magnetic resonance spectroscopy
**$SCN^-$-** Thiocyanate
**TPO-** Thyroid peroxidase
**UV/Vis-** Ultraviolet/Visible spectroscopy

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## Abstract

Lactoperoxidase is an essential component of the defense mechanism of mammalian secretory fluids. It is a heme protein that generates a non-specific, unidentified oxidized species during the catalysis of thiocyanate to hypothiocyanate, which prevents viruses, bacteria and other microbes from invading a mammalian cell. Both the unusual mechanism and structure of this enzyme will be discussed.

# Introduction

Lactoperoxidase is the solution to the evolutionary problem of invading microbes. That is why mammals include lactoperoxidase in their secretory fluids, such as tears, milk and cervical fluid. An oxidizing species is generated during the catalysis of $SCN^-$ that wreaks havoc with non-mammalian invaders. The general enzymatic reaction for peroxides is rather simple:

$$LPO + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$
$$\text{Compound I} + AH \rightarrow \text{Compound II} + A^\bullet \quad (2)$$
$$\text{Compound II} + AH \rightarrow LPO + A^\bullet + H_2O \quad (3)$$

Where A is a generic substrate. Compound I is an Fe(IV) = O with a radical spread out on the porphyrin ring [3], and Compound II is the same, except the radical is passed to the substrate. When thiocyanate, the normal substrate, is used, the sum of the reactions is thus:

$$H_2O_2 + SCN^- \xrightarrow{LPO} H_2O + OSCN^- \quad (1)$$

A second water is not formed, because $SCN^-$ is reduced by the extra electrons.

LPO is a single strand polypeptide, 612 amino acids long [1] and weighs approximately 78,000 daltons. The structure of LPO is unusual in that the prosthetic heme group is covalently linked to the protein backbone by a pair of ester linkages to carboxylic acids in the active site of the enzyme [2]. Other peroxidase enzymes, such as eosinophil peroxidase, thyroid peroxidase and myeloperoxidase share this substituted heme, but only myeloperoxidase is more oxidizing than LPO. Several heme active sites are shown below in Figure 1, adapted from [2].

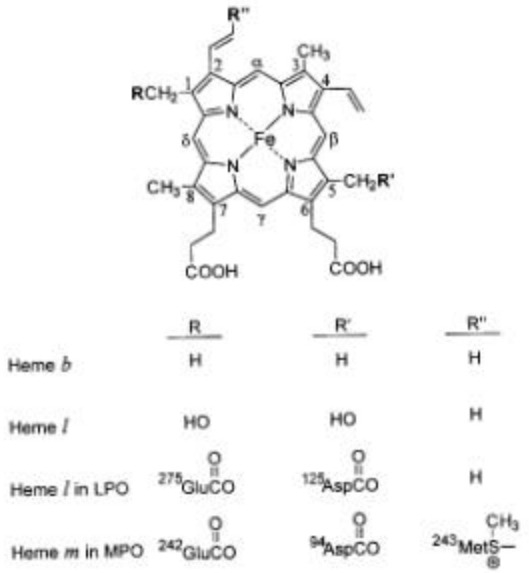



**Figure 1-** The heme active site in various biological systems. Heme *l* in LPO is substituted twice by the protein backbone, at the 1- and 5-methylene sites. Heme *m* in MPO is substituted in the same positions but with an additional sulfonium linkage at the 2-vinyl position to a methionine found in the protein backbone.

The mechanism of heme incorporation is not known, but several theories have been discussed, and will be introduced later. Free radical mechanisms are suspected to occur in both the substitution of the heme and during oxidation of thiocyanate. EPR spectroscopy has detected a protein-based radical upon reaction of the protein with $H_2O_2$ [3]. In addition, a free radical intermediate of thiocyanate oxidation, which is suspected to cause the antimicrobial activity of LPO, has been trapped and its structure theorized [4]. This is an important finding because neither the products nor reactants are present in large enough concentrations to inhibit microbial growth.

## Isolation of Lactoperoxidase

The isolation of LPO from bovine milk is an arduous process, but is the easiest method of obtaining a peroxidase enzyme, since other sources of peroxidases include tears, sweat and saliva, which can not be collected *en masse* as easily as milk [5]. Approximately 0.13 to 0.27 grams of LPO could be extracted from 20 L of unpasteurized milk. LPO content can be confirmed by UV/Vis spectroscopy. If a spectrum has an $R_z$

value ($A_{408}/A_{280}$) greater than 0.70, LPO is present. This ratio verifies the presence of LPO, due to the heme absorption in the Soret band (408 nm) and the aromatic protein backbone absorption (280 nm). A typical spectrum is shown below, in Figure 2.

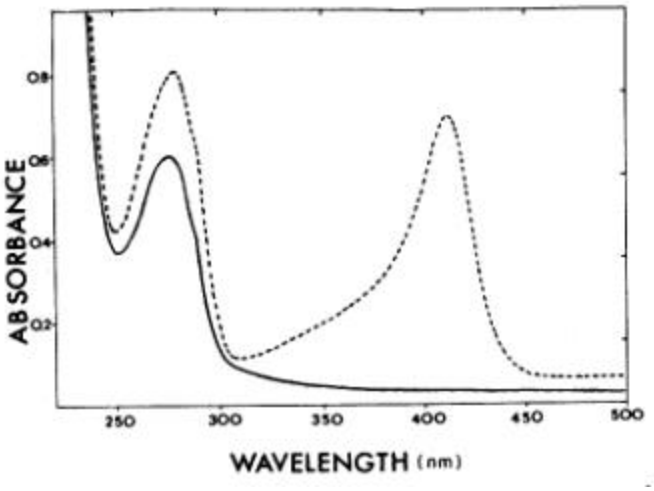



**Figure 2-** Absorption spectra of heme(----) and non-heme (___) LPO. The presence of the heme group is responsible for the absorption in the Soret band. The pathlength is 1 cm, and concentrations are 0.7 mg/mL. $\varepsilon_{280}$ is 85,000 $M^{-1}$ $cm^{-1}$, while $\varepsilon_{412}$ is 100,000 $M^{-1}$ $cm^{-1}$. Adapted from [6].

## Detection of Lactoperoxidase

LPO has a distinctive MS signal, as well as an EPR signal. These are pictured in Figures 3 and 4, respectively.

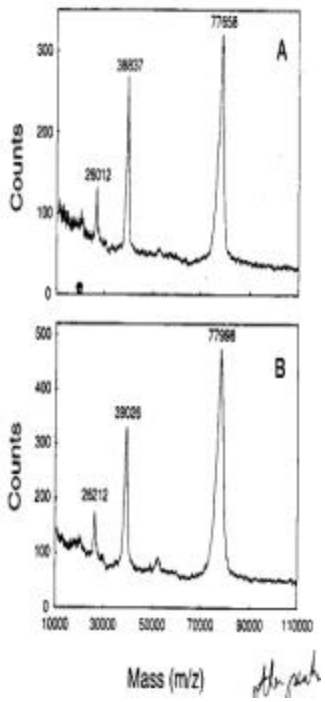



**Figure 3-** MALDI mass spectrum of LPO. The mass labels correspond to the $[M + H]^{1+}$, $[M + 2H]^{2+}$, and $[M + 3H]^{3+}$, respectively. Adapted from [6].

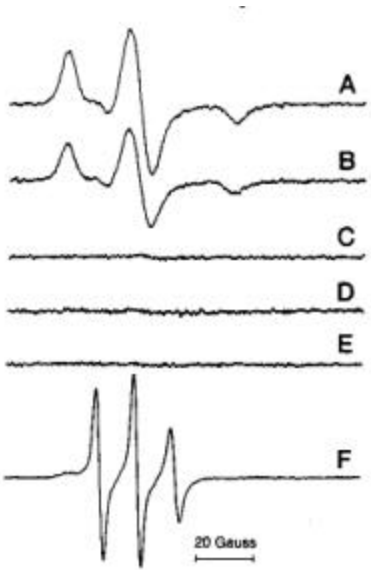
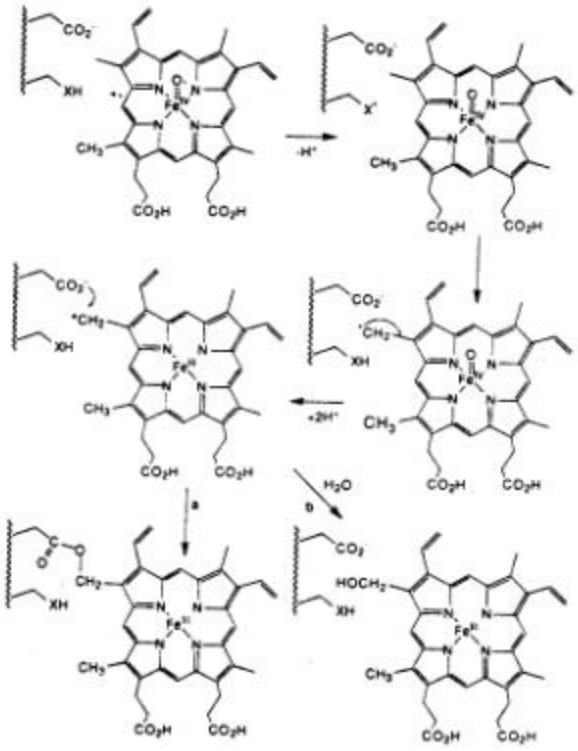



**Figure 4-** EPR spectra obtained from the reaction of bovine LPO with $H_2O_2$ in the presence of DBNBS, a spin trap. The g-value is 2.0065. Adapted from [3].

The EPR signal was obtained by trapping the radical on the protein in Compound I, and is believed to be based on a nearby tryptophan [3]. Other work suggests it may be based on another amino acid, a phenylalanine [9]. The spin trap, DBNBS, is especially adept at picking up carbon-centered radicals, so other spin traps are being used to attempt to find the radical as it progresses through the mechanism [12].

A crystal structure of LPO has not yet been obtained, so the x-ray structure of MPO [7] is used as a model. These proteins only share 58% sequence identity [8], but structurally important residues, such as carboxylate linkages, $Ca^{2+}$ binding sites, proximal and distal histidines, and many others, are conserved. In fact, within a 7Å radius of the heme active site, there is an 84% sequence similarity [9]. Structural studies are ongoing, and hopefully will determine the mechanism of substitution.

The most accepted mechanism of substitution was proposed by Ortiz de Montellano, *et al* and requires both carboxylate and aromatic amino acids in the vicinity of the substituted sites. Scheme 1 shows the proposed mechanism of substitution.



**Scheme 1-** Proposed mechanism of substitution of LPO. Upon addition of $H_2O_2$ to the heme, a nearby aromatic amino acid is oxidized. It abstracts an H from the 1-methylene site, leaving a porphyrin radical. Iron is oxidized, with the removal of water, leaving a carbocation near the carboxylic acid. They form a covalent bond, and the substituted heme is formed. Adapted from [10].

In a recent study [9], the amino acid residues near the heme were examined. Obviously, Asp and Glu residues were studied because they could bond to the porphyrin. Tyr, Phe and Trp were studied because they are logically X, the radical stabilizer. Evidence for Tyr radicals exists for Photosystem II and Ribonucleotide Reductase, so a search was conducted for a conserved Tyr residue near the 1- and 5-methylene site. The closest Tyr residues near the active site in MPO are over 9Å distant, and too far away to be involved. Phenylalanine residues are within 3.55Å of the 1-methylene site and 4.62Å from the 5-methylene site. Their close proximity suggests that both substitutions could be stabilized by a radical present on any of the nearby phenylalanine groups. Phe is not expected to exist as a radical due to the high reduction potential of the $Phe^{\cdot}$, $H^{+}$/Phe couple (1.6 to 1.9 V) [11], but is the only close aromatic group in this system.

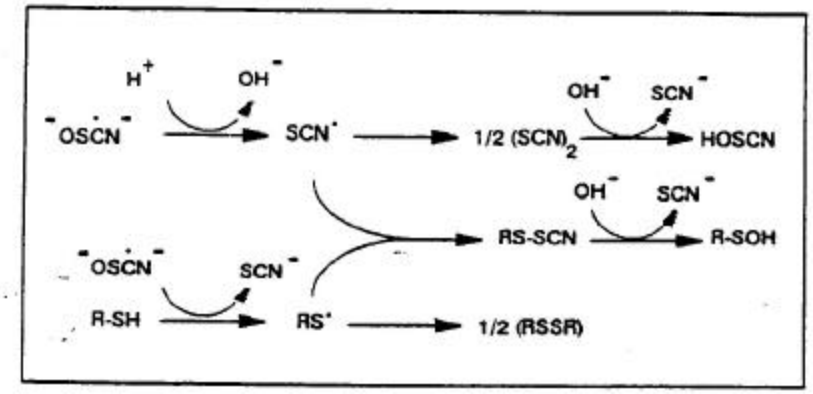
From these results, it has been proposed that Phe has an important role in the function of LPO. Its role has not been defined, but there are two possibilities. It may provide radical stabilization, either during the substitution of the porphyrin or during oxidation of $SCN^{-}$, or both. A second possibility is that it controls the redox potential of the heme environment.

## Reactions of Lactoperoxidase

The substitution of the heme active site is suspected to increase the reduction potential of LPO. It is large enough to catalyze the iodination of tyrosine [8], as well as oxidize $SCN^{-}$, a psuedohalide. Table 1 shows the various oxidizing potentials of substrates, and which peroxidase will catalyze their reaction.

| **Substrate** | **E° (vs. SHE)** | **Catalyzed by** |
|---|---|---|
| $Cl^-$ | -1.36 V | MPO |
| $Br^-$ | -1.07 | MPO, EPO |
| $SCN^-$ | -0.77 | MPO, EPO, LPO, TPO |
| $I^-$ | -0.54 | MPO, EPO, LPO, TPO |

The unknown, antimicrobial intermediate of LPO is also another hot topic of study. Several mechanisms have been proposed, but none have captured a radical which can be proven to be an intermediate. Lovaas obtained an EPR signal [4], but it was at non-physiological levels of reactants. High levels of $H_2O_2$, KSCN, and DMPO were used, in addition to LPO. The structure was hypothesized to be $^-OSC{\cdot}N^-$, a dianion C-centered radical, to be the intermediate, based on spectra. Despite using the same concentrations and amounts of solutions, others [9] were unable to replicate his results. His proposed mechanism is pictured as Scheme 2.




**Scheme 2-** Possible breakdown products of the proposed intermediate, $^-OSC{\cdot}N^-$. Adapted from [4].

Future experiments on LPO will determine the radical intermediate by utilizing flow systems [12]. A myoglobin radical has been detected [11] in a flow system, so similar experiments with LPO as the enzyme will be tried. A significant downside to this method is the amount of enzyme required. Other future experiments include freeze

quench methods, wherein the reaction will be halted at specific time periods by an isopentane slurry with subsequent EPR examination [12]. Hopefully, trapped intermediates will allow mechanistic steps to be characterized, and the antimicrobial agent produced by LPO will be discovered.

## Conclusion

A free radical mechanism for both the substitution of LPO heme and the oxidation of $SCN^-$ have been proposed. Computer modeling has determined the most likely candidate for stabilization of the radical to be nearby Phe residues, an unusual choice due to the high reduction potential of that amino acid. The detection of these radicals has so far been unsuccessful, but new methods, such as flow systems and freeze quench techniques will be employed. Hopefully, the antimicrobial species produced by LPO will be discovered by these methods.

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