Review Article
Lactoperoxidase: structural insights into the function, ligand binding and inhibition

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Abstract: Lactoperoxidase (LPO) is a member of a large group of mammalian heme peroxidases that include myeloperoxidase (MPO), eosinophil peroxidase (EPO) and thyroid peroxidase (TPO). The LPO is found in exocrine secretions including milk. It is responsible for the inactivation of a wide range of micro-organisms and hence, is an important component of defense mechanism in the body. With the help of hydrogen peroxide, it catalyzes the oxidation of halides, pseudohalides and organic aromatic molecules. Historically, LPO was isolated in 1943, nearly seventy years ago but its three-dimensional crystal structure has been elucidated only recently. This review provides various details of this protein from its discovery to understanding its structure, function and applications. In order to highlight species dependent variations in the structure and function of LPO, a detailed comparison of sequence, structure and function of LPO from various species have been made. The structural basis of ligand binding and distinctions in the modes of binding of substrates and inhibitors have been analyzed extensively.

Keywords: Lactoperoxidase, lactoperoxidase system, mammalian heme peroxidases, antimicrobial, structure

LPO: a member of mammalian heme peroxidase family

Lactoperoxidase (LPO) (EC 1.11.1.7) is a heme-containing chain glycoprotein found in milk and other exocrine secretions such as saliva, tears and airways. LPO binds a covalently linked heme prosthetic group, a derivative of protoporphyrin IX in its catalytic center. It belongs to the family of mammalian heme-containing peroxidase (XPO) enzymes. Apart from LPO, this family of proteins includes myeloperoxidase (MPO), eosinophil peroxidase (EPO), and thyroid peroxidase (TPO). Though the members of this family have a similar function as plant and fungal peroxidases [1-3], they demonstrate differences from the latter in the mechanism of ligand binding. Also, while the prosthetic heme group in mammalian peroxidases is bound to the protein through covalent bonds [4-9], there is a significant absence of covalent bonds in fungal and plant peroxidases [10-14].

All the members of XPO family catalyze a similar reaction in multiple steps:

H_2O_2 + reduced acceptor → oxidized acceptor + H_2O
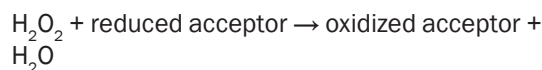
Through this reaction, these proteins convert the halides and pseudohalides to hypohalous and hypothiocyanous acids [15-19]. The products of this reaction are potent oxidants which show significant biological actions. The four members of the XPO family show similarities in their overall structures and functions. However, they show significant differences in other aspects (**Table 1**). Though both MPO and EPO are heterodimeric glycoperoteins [20-23], LPO and TPO exist as monomers [24-27]. While MPO is abundantly expressed in the neutrophils [28, 29] and EPO is primarily found in eosinophils [30, 31], LPO is found in milk, saliva, tears and other exocrine solutions [32-35] and TPO is a membrane bound protein primarily localized in the thyroid gland [36, 37]. The proteins also differ in the way the heme group is bound to them in the catalytic centre. While the heme group is bound to MPO through three covalent bonds, there are only two covalent linkages binding the heme to the LPO, EPO, and TPO. Within the XPO

Table 1. Comparison of Characteristics of proteins from Mammalian Heme Peroxidase (XPO) family

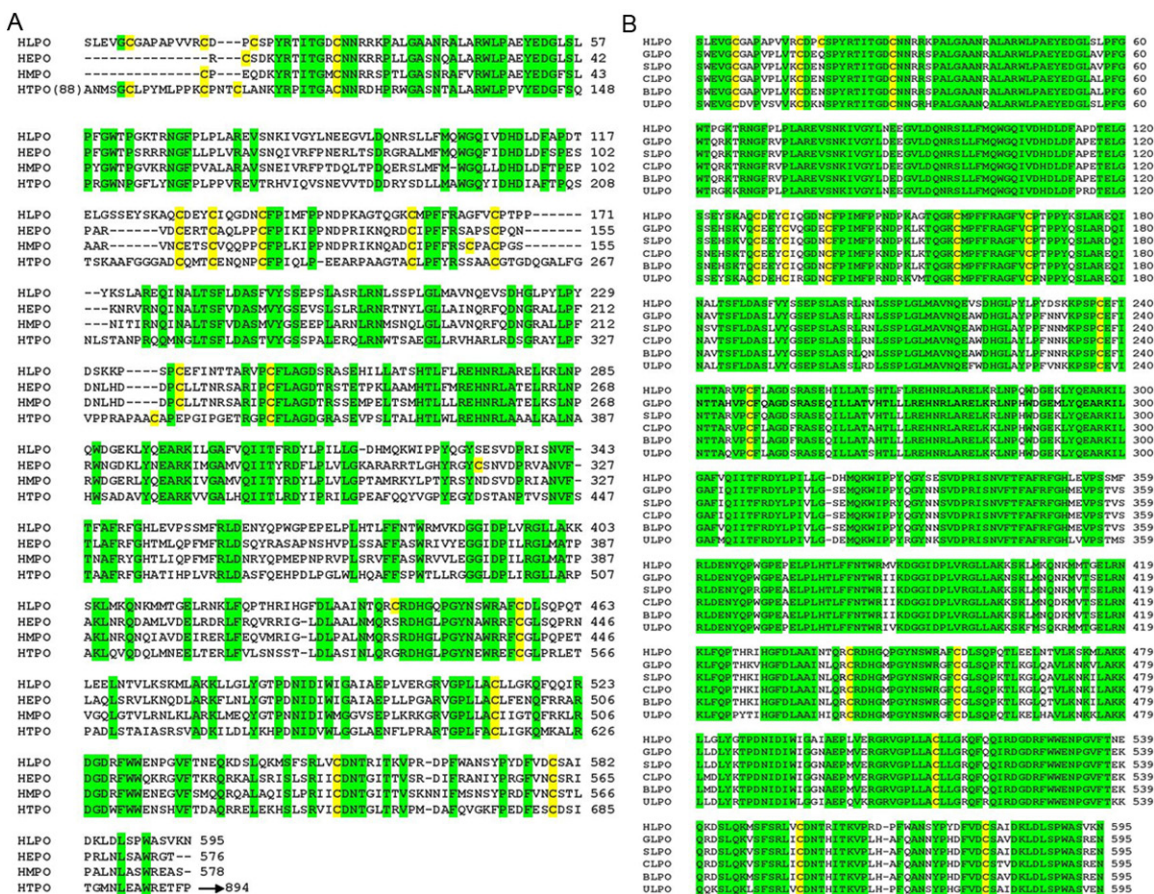|	Lactoperoxidase	Myeloperoxidase	Eosinophil peroxidase	Thyroid Peroxidase
Found In	Milk, Saliva and airways	Neutrophils	Eosinophils	Thyroid gland
Chains	1	2	2	1
Number of covalent bonds of Heme	2	3	3	3
Crystal Structure Known	Known	Known	Not known	Not known

Figure 1. A: Sequence comparison of four mammalian human peroxidases, HLPO, HMPO, HEPO, and HTPO is shown. The numbering schemes original to individual proteins have been indicated. The molecular mass of HTPO is of the order of 100 kDa, and its sequence is extended on both sides of N and C-termini of the other three peroxidases. Cys residues are indicated in yellow. The identity in the sequences is highlighted in green. B: Sequence comparison of LPOs from human (HLPO), goat (GLPO), sheep (SLPO), cow (CLPO), buffalo (BLPO), and camel (ULPO). The residues have been numbered from 1 to 595. Cys residues are indicated in yellow. The identity in the sequences is shown in green.

family, the crystal structures of two members, MPO and LPO have been determined [38-46]. Over the past few years, it has been observed that all the members of XPO family are implicated in a number of host defense and pathology of diseases. However, the structure-function interrelationships of these proteins are still not completely understood, primarily because till 2008, only the structure of MPO was determined. The complete understanding of their molecular structures and their implication on their function would lead to opening up for vistas to exploit these proteins as effective drug targets.

LPO and LPO-system (LPS): discovery and early days

LPO was named so because it was isolated from milk in crystalline form for the first time
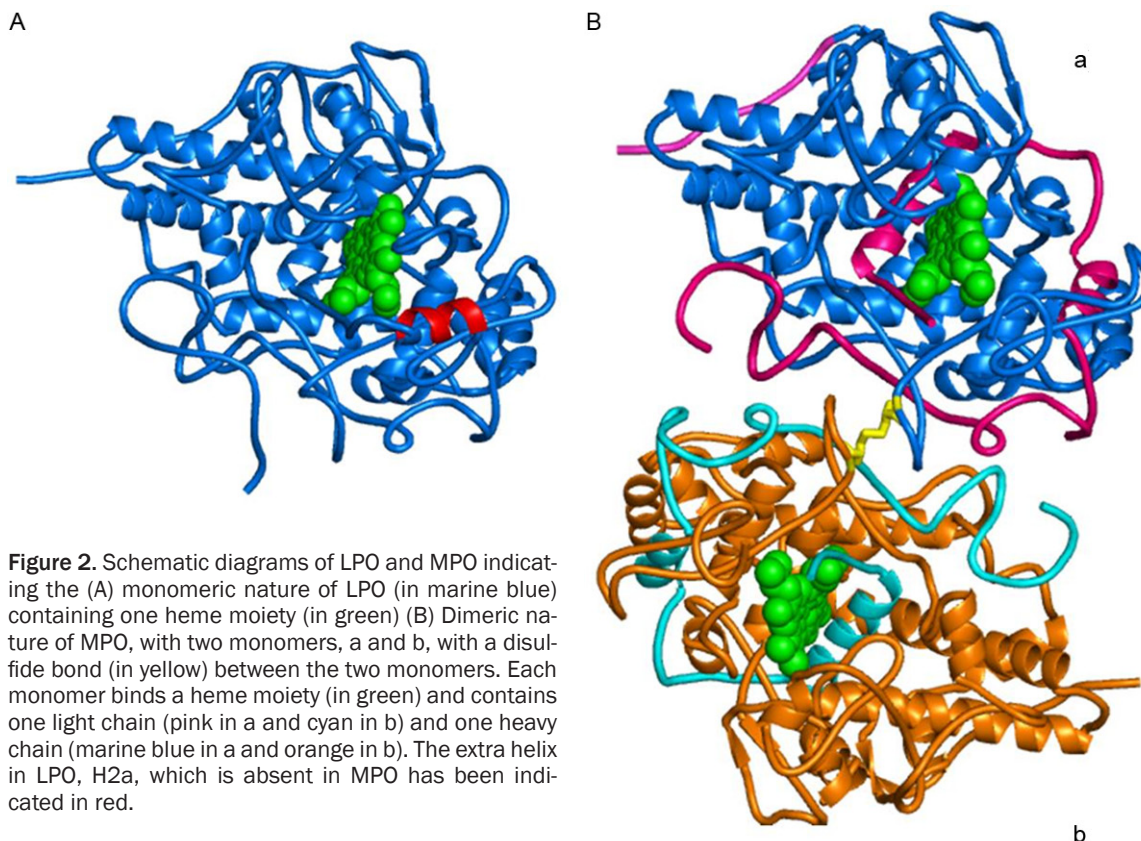
A

B

Figure 2. Schematic diagrams of LPO and MPO indicating the (A) monomeric nature of LPO (in marine blue) containing one heme moiety (in green) (B) Dimeric nature of MPO, with two monomers, a and b, with a disulfide bond (in yellow) between the two monomers. Each monomer binds a heme moiety (in green) and contains one light chain (pink in a and cyan in b) and one heavy chain (marine blue in a and orange in b). The extra helix in LPO, H2a, which is absent in MPO has been indicated in red.

[47]. LPO was later purified using ion exchange chromatography by Morrison et al [48]. The protein was also found to be secreted from other glands, such as lacrimal glands, hardenian glands [34] and salivary glands [35]. Over the next few years, this enzyme was characterized in terms of its antibacterial function. LPO was demonstrated to inhibit the growth of lactic acid streptococci in the presence of hydrogen peroxide (H_2O_2) and thiocyanate (SCN^-) [49, 50]. The importance of the presence of H_2O_2 was discovered in 1962 as it was seen that that LPO needs H_2O_2 to inactivate bacteria [51]. In 1963, it was established that LPO mediates its antibacterial action through an inhibitory system named LPO system (LPS) that consists of LPO, SCN^- and H_2O_2 [52, 53], where LPO and SCN^- are naturally present in milk and H_2O_2 is generated by bacteria [53]. LPO was also found to inhibit other bacterial strains such as *Staphylococcus aureus, Streptococcus faecalis*, *Escherichia coli* and some similar pathogens [54-58].

In the next few years, LPS was shown to inhibit the growth of various other bacteria like *Salmonella typhimurium*, *Pseudomonas aeruginosa* [56, 59] and *Streptococcus agalactiae* [60]. It was also demonstrated to show a bactericidal effect on *Plasmodium falciparum* [61]. It enhanced the thermal destruction of *Listeria monocytogenes* and *Staphylococcus aureus* [62, 63]. LPS, in combination with high hydrostatic pressure was shown to combat microbial action effectively [64, 65]. LPS was also found to be responsible for degrading carcinogens and protecting cells against peroxidative action [66]. It was found that apart from the antibacterial action, LPS is an effective antiviral [67, 68] and antifungal agent [69-71].

Applications of LPS in industry and medicine

Due to its broad spectrum antibacterial properties, LPS was explored for its potential role as an agent to preserve foods and milk, and its use in medicine. Initially, several reports established. LPS as a feasible procedure for controlling the growth bacteria in raw milk at refrigeration temperatures [62, 72, 73] as well as pasteurized milk [74]. It was shown that LPS can be successfully utilized to increase the shelf life of
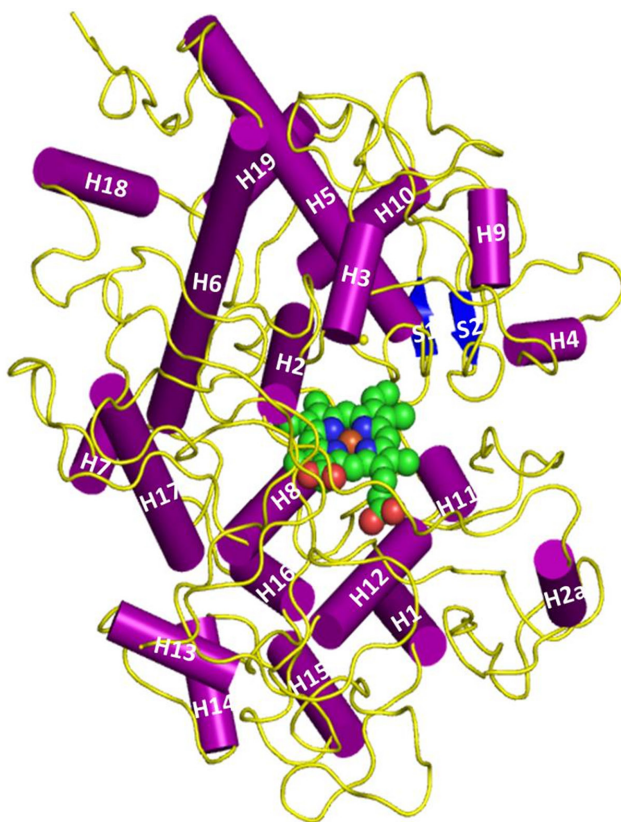
Figure 3. Molecular structure of LPO. Purple cylinders indicate α-helices and blue arrows indicate β-strands. Heme group in green, is shown in the centre as a ball and stick model.

milk in the tropics [75]. LPS, along with other preservatives was demonstrated to prevent the growth and survival of *Salmonella enteritides* in minimally processed fruit and vegetable products [76]. LPS has been used to curtail the growth of *Enterobacter sakazakii*, which grows in reconstituted infant formula and is responsible for several neonatal pathologies [77]. LPS was also shown to prevent the development of bacterial colonies which cause post-harvest diseases in mangoes [78, 79]. LPS, in combination with nisin, was reported to promote the preservation of fish [80]. It was found to have a strong inhibitory action on *Escherichia coli* O157:H7 and *Listeria monocytogenes* on ground beef [81] and was also found to inhibit the growth of native pseudomonads on beef cubes inoculated with bacteria but did not prevent the development of native lactic acid bacteria [82]. In a similar study, it was reported that LPS was able to inhibit the growth of *Salmonella enterica* and *Escherichia coli* O157:H7 on roasted turkey [83].

The potential applications of LPS in dentistry started becoming evident as it was found that LPS was effective in the prevention of dental caries and plaque accumulation [84, 85]. It was also shown that toothpaste containing LPS reduced the incidence of cariogenic bacteria in children [86]. LPS was also found to inhibit several strains of clinically active gastric pathogen, *Helicobacter Pyroli* [87]. LPS was also found to be effective in the bacterial clearance of the airways, indicating a possible application in patients suffering from cystic fibrosis [88-90].

LPO: sequence analysis

The sequence comparison of human LPO (HLPO) with human MPO (HMPO), human EPO (HEPO) and human TPO (HTPO) revealed a sequence identity of 57%, 58% and 46% respectively (**Figure 1A**). The two main regions in the sequences of these proteins which showed maximum differences are the N-terminal region and at position 120 of LPO. In the N-terminal region, LPO and TPO contain extra 14 residues which contain the first cysteine residue found in these proteins. However, this stretch is absent in MPO and EPO. MPO sequence begins with a cysteine residue, which is Cys1 in MPO and Cys16 in LPO. However, EPO sequence starts with an Arginine (Arg) residue and does not contain that cysteine residue (Cys6 in LPO and Cys1 in MPO). Additionally, a hexamer which is present in LPO and TPO (Ser121-Lys126 in LPO and Ala212-Gly217 in TPO) is completely absent in MPO and EPO. Interestingly, this is the hexamer that is truncated in the MPO which leads to the presence of a heavy chain and a light chain in each equal half of MPO dimer. In this respect too, EPO shows similarity with MPO and lacks the hexamer while TPO, like LPO, contains the hexamer, even though the sequence of the hexamer in LPO (Ser-Ser-Glu-His-Ser-Lys) is very different from that in TPO (Ala-Ala-Phe-Gly-Gly-Gly).

TPO seems to be most different from other heme peroxidases, probably because this is the only membrane protein amongst all, and it contains several insertions which are absent in other XPOs, for instance, a tripeptide containing the second cysteine at position 103, an octapeptide at position 262, a proline (Pro)
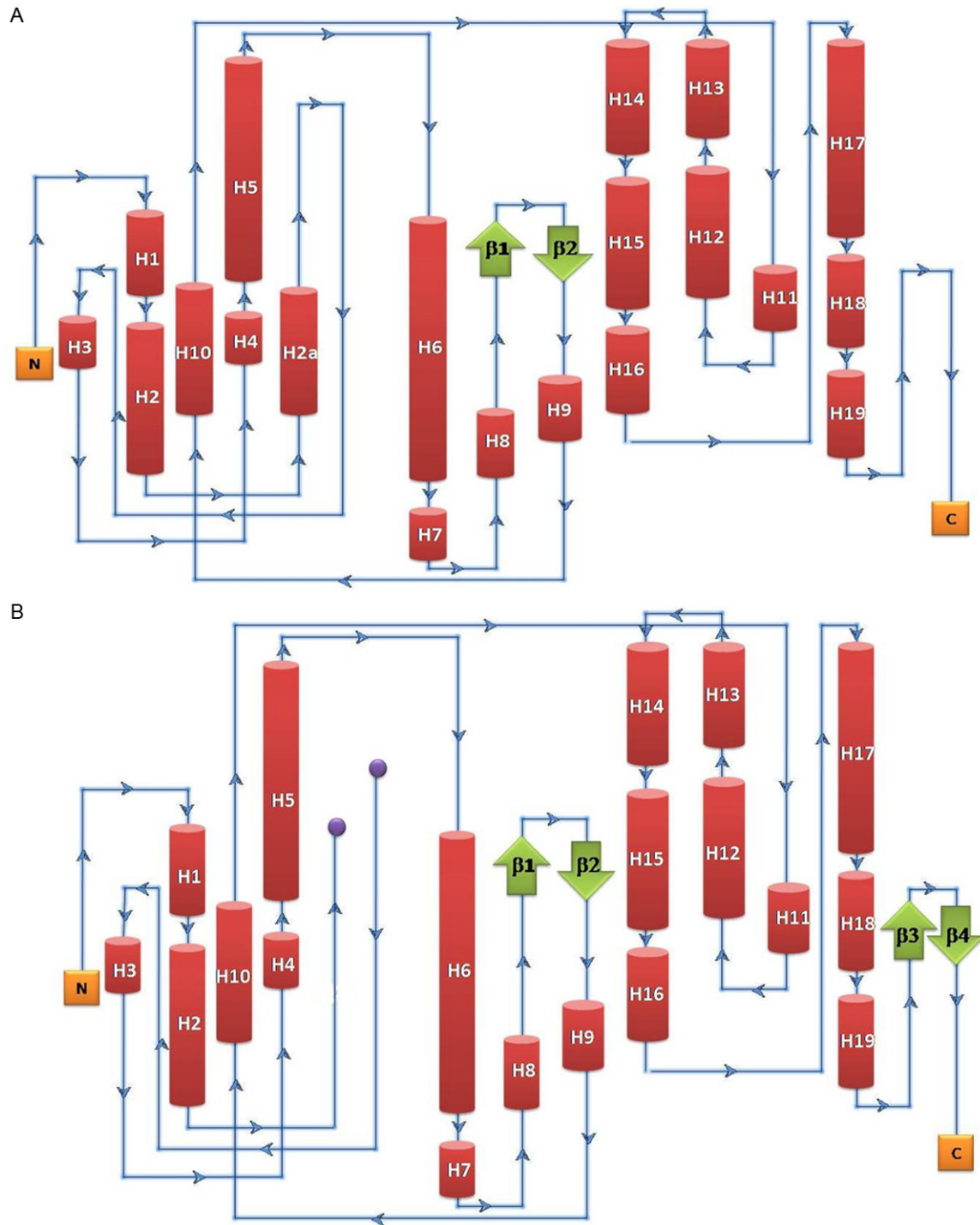
Figure 4. Topological diagram of (A) LPO and (B) MPO, showing the crucial differences between the two proteins.

residue at 330, a tripeptide (Gly-Asn-Pro) at position 340 and several other minor insertions, all of which are absent in other XPO proteins.

While MPO and EPO contain 15 cysteines, TPO contain 14 cysteine residues. LPO from all spe-

cies contain 15 cysteine residues, with the exception of HLPO which contains 16 cysteines (**Figure 1B**). In the structure of MPO, there are 14 cysteines, which form 6 intramolecular disulfide bonds (Cys1-Cys14, Cys115-Cys125, Cys119-Cys143, Cys221-Cys232, Cys440-Cys497, Cys538-Cys564) and 1 intermolecular

Table 2. Secondary Strucure Elements of Lactoperoxidase in comparison with Myeloperoxidase

Helices		
Helix	Lactoperoxidase	Myeloperoxidase
H1	75-83	61-69
H2	98-111	84-97
H2a	124-132	-
H3	197-203	181-187
H4	236-240	220-224
H5	260-283	244-267
H6	289-317	273-301
H7	321-324	303-309
H8	341-353	326-338
H9	383-387	368-373
H10	391-401	376-385
H11	415-419	400-404
H12	433-444	417-428
H13	449-456	433-440
H14	463-471	448-454
H15	475-483	458-468
H16	492-498	476-482
H17	509-525	493-508
H18	538-546	522-530
H19	549-556	533-539
β-strands		
β1	356-359	342-344
β	373-375	358-360

disulfide bond (Cys153-Cys153). Out of the six intramolecular disulfide bonds present in the protein, only one is present in light chain (Cys1-Cys14) and the rest five are present in the heavy chain. Cys153 in MPO has been substituted with a glycine residue in LPO (Gly163) and hence, there is no dimerization in case of LPO. In the case of all LPOs whose structures have been determined, that is bovine, caprine, sheep and buffalo, the 15 cysteines form 7 intrachain disulfide bonds; Cys6-Cys167, Cys15-Cys28, Cys129-Cys139, Cys133-Cys157, Cys237-Cys248, Cys456-Cys513, and Cys554-Cys579, while only Cys441 exists as a free cysteine. In case of HLPO, it would be convenient to presume that Cys18 would form an eighth disulfide bond with Cys441. However, as seen from the LPO structures determined from other species, the position of Cys18 residue is too far apart from the Cys441 residue to form a disulphide bond. While Cys441 is buried inside the protein, the 18th residue (Asn in other LPOs) is conspicuously on the surface of the protein. Hence,

it remains to be seen if the Cys18 in case of HLPO would be involved in its dimerization, as Cys153 in HMPO which makes an interchain disulfide bond is also present on the surface of the monomer leading to the formation of disulfide bond with another MPO monomer.

In case of the EPO and TPO too, the Cys153 of MPO has been substituted with other residues, i.e., Alanine (Ala) in EPO and Serine (Ser) in TPO respectively. While this explains why TPO is also a monomer like LPO, the same cannot explain why EPO lacks cysteine at that position and still exists as a dimer. Hence, there has to be another disulfide bond in EPO that binds its two monomers. At position 315, there is a cysteine residue (Cys315) in EPO which is absent in all other XPOs. Though the crystal structure of EPO is not known, it can be observed from the crystal structures of MPO and LPO that this cysteine would be present on the surface, and hence, it is speculated that this residue may be making an interchain disulfide bond leading to dimerization in the case of EPO.

Through the sequence analysis, it is easy to understand why MPO binds heme moiety with three covalent linkages, while all other XPOs bind it with two covalent bonds. The reason is the presence of a methionine residue at position 243 (Met243) which provides an interaction between the sulfonium ion and the terminal β-carbon of the vinyl group on pyrrole ring A. This methionine is substituted with histidine (His), threonine (Thr) and valine (Val) in LPO, EPO and TPO respectively (**Figure 1A**).

The sequence comparison of LPOs from various species like, human (HLPO), bovine (CLPO), buffalo (BLPO), goat (GLPO), sheep (SLPO) and camel (ULPO) was analyzed (**Figure 1B**). Among each other, LPOs from various species show a high sequence identity, for instance the sequence identity of HLPO with CLPO, BLPO, GLPO, SLPO and ULPO is 85%, 85%, 84%, 84% and 85% respectively. CLPO, BLPO, GLPO and SLPO share a very sequence identity ranging from 95-98%. Only ULPO seems to be relatively different from other LPOs as it does not show more than 85% identity with any other LPOs.
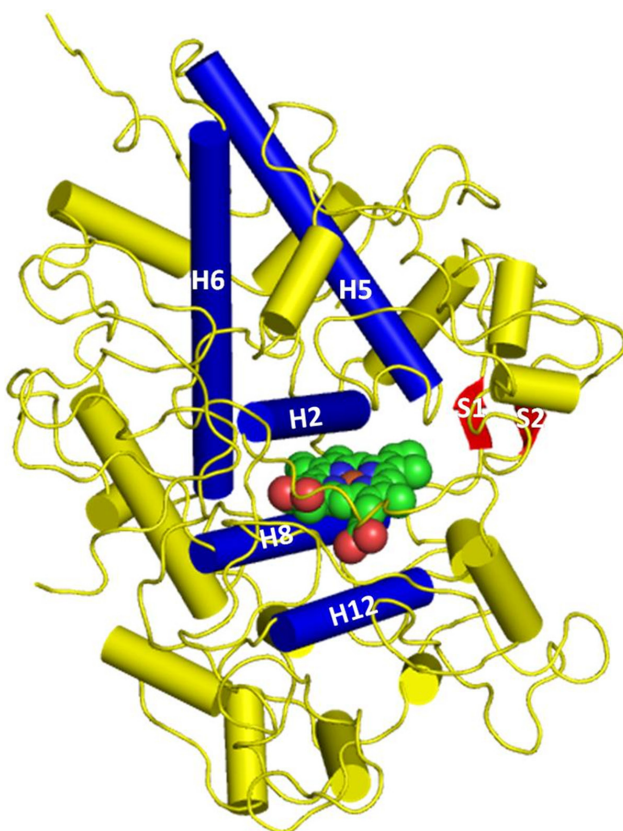
Figure 5. Schematic diagram of molecular structure of LPO (yellow) with core helices H2, H5, H6, H8 and H12 indicated in blue, and β-strands, S1 and S2 (in red), holding the heme moiety (in green) in the catalytic centre.

LPO: structural features

While the crystal structure of canine MPO was reported in 1992 [22], followed by the structure of HMPO in 2000 [39], the elucidation of the first three-dimensional structure of LPO was reported in 2008 [25]. This was the first report of LPO structure from any species and second structure of a heme mammalian peroxidase. Consequently, the reports on the structures of LPO from other species and their complexes, that is CLPO in complex with hypothiocyante [25], aromatic ligands [41, 45], isoniazid [46], inorganic substrates [44] and BLPO in complex with thiocyanate [42] were reported.

The primary structure of LPO from all the species is a single chain monomer of 595 residues. In accordance with the primary structure, the X-ray crystal structure of LPO has revealed a globular single chain protein of 595 residues containing the heme moiety in the catalytic center (**Figure 2A**). On the other hand, MPO

was found to be a dimer which contains two subunits connected to each other by an interchain disulfide bond. Each subunit binds a heme moiety and contains a light chain and a heavy chain of 108 and 466 amino acids respectively (**Figure 2B**). This unusual structure of MPO was speculated to have arisen from post-translational excision of a hexapeptide from a single polypeptide precursor [91, 92]. The over-all structure of LPO is oval shaped, open on one side by a substrate diffusion channel which leads to the interior of the heme cavity and completely closed on the other side by a tight criss-cross of α-helices. The main features of the structure of LPO are the twenty α-helices (H1 to H19) which make the core of the structure; two short anti-parallel β-strands (S1 and S2) and the central heme cavity which can be divided into proximal and distal cavities (**Figure 3**).

Secondary structural organization in LPO

α-helices are the secondary structural elements that predominate the structure of LPO. There are 20 alpha helices in the structure, named as H1 (75-83), H2 (98-111), H2a (124-132), H3 (197-203), H4 (236-240), H5 (260-283), H6 (289-317), H7 (321-324), H8 (341-353), H9 (383-387), H10 (391-401), H11 (415-419), H12 (433-444), H13 (449-456), H14 (463-471), H15 (475-483), H16 (492-498), H17 (509-525), H18 (538-546) and H19 (549-556). There are only two short β-strands in the whole structure, named as S1 (356-359) and S2 (373-375).

The comparison of secondary structure elements of LPO and MPO is shown in **Figure 4**. The overall secondary structure elements of both the molecules are similar (**Table 2**). However, one helix, H2a (124-132) which is present in LPO, is absent in MPO. The reason for this could be the fact that myeloperoxidase is truncated in this region which could lead to loss of secondary structure elements (**Figure 4A** and **4B**).

Heme cavity

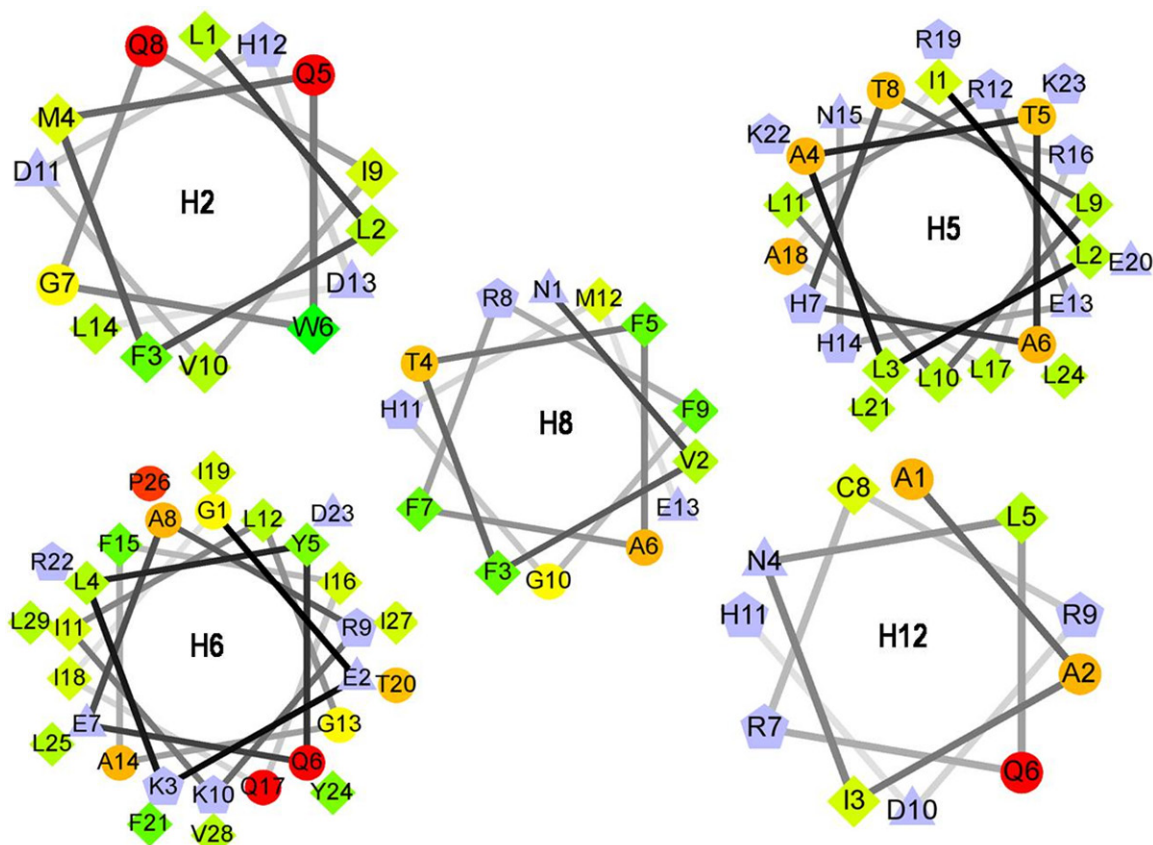The heme molecule is bound in a heme cavity which is deeply buried inside the structure of

Figure 6. Helical wheel representation of the core helices of LPO. The hydrophilic residues are shown as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. Hydrophobicity is color coded as well: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to low hydrophobicity, coded as yellow. Hydrophilic residues are coded red with pure red being the uncharged residues, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue. (The plots were made using the software created by Don Armstrong and Raphael Zidovetzki. Version: 0.10 p06 12/14/2001 DLA modified by Jim Hu).

LPO. Five helices (H2, H5, H6, H8 and H12), also called the 'core helices' are arranged in a unique structure which gives the required stereochemistry and shape for the heme to bind in the heme cavity (**Figure 5**). The floor of the core heme cavity is constructed by two helices, H8 and H12 and the roof of the heme cavity is supported by two long vertically placed helices, H5 and H6 with a short helix, H2 in between. The helices H8, H2 and H5 run almost parallel to the large heme ring, so that the heme ring is tightly sandwiched between H2 and H8. On the other hand, H5 and H6 are oriented perpendicular to the other three core helices as well as the heme ring, and along with helix H2, they form a prismatic support for the heme ring at the top of the heme cavity. The two β-strands, S1 and S2 form the upper side wall of the opening to the heme cavity.

It is significant to note that these most of these helices are highly hydrophobic helices and their hydrophobic residues are oriented towards the hydrophobic heme ring, as is evident from their helical wheel representations (**Figure 6**). This shows that the architecture of the heme cavity has been constructed by proper positioning of the five core helices.

A heme moiety consists of a heterocyclic ring or porphyrin which is essentially four pyrrole molecules linked to each other, holding one iron atom in its center. Each pyrrole ring is designated as A, B, C and D. The inner nitrogen atoms of the four pyrrole rings are aligned in order to make coordinate bonds with the iron in the center of the ring. In the LPO structure, a conserved histidine residue, His351 from the helix H8 makes the fifth coordination bond with the iron atom, while a highly conserved water
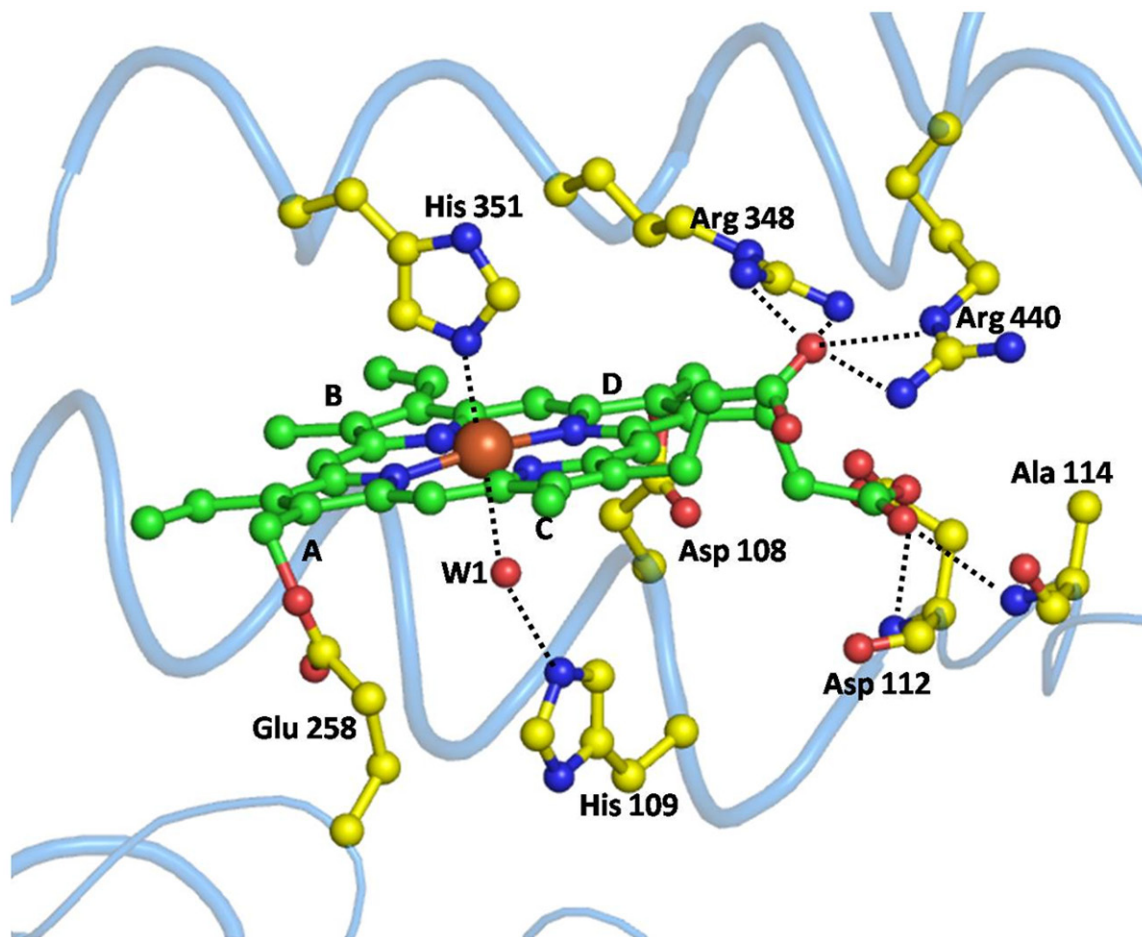
Figure 7. Interactions of the heme moiety (green) with the residues of LPO (yellow). The heme iron is indicated in orange. The covalent interactions with Asp108 and Glu258 are indicated as solid bonds. The hydrogen bonds with His351, Arg348 and Arg440, Ala114, Arg112 and W1 are indicated as dashed lines.

molecule, designated as W1 makes the sixth coordinate bond with the iron, so that the octahedral group of six ligands is complete. This stereochemistry results in tweaking of the planarity of the heme moiety so that while the pyrrole rings A, C and D are planar, the pyrrole ring B is somewhat shifted from the overall plane of the rest of the heme moiety. The outer methyl groups of pyrrole rings A and C form ester bonds with the carboxyl groups of Glu258 and Asp108, respectively (**Figure 7**). While the ring C propionate interacts with Asp112 Oδ2 and Ala114 N, the carboxyl group of the pyrrole ring D propionate interacts with the guanidinium groups of Arg348 and Arg440.

The heme cavity of LPO is asymmetrically divided into two regions, the distal heme cavity and the proximal heme cavity (**Figure 8**). The proximal heme cavity is considerably smaller, as compared to the corresponding distal cavity. It consists of His351 of helix H8 which interacts with the iron atom of the heme. Consequently, the iron position is shifted slightly toward the proximal site. The distal heme cavity, which is larger than the proximal heme cavity, consists of three aspects; the substrate diffusion channel, the substrate binding site and a conserved water network.

The substrate diffusion channel consists of a long channel, approximately 22Å in length and 10Å in diameter. It is open on one end to facilitate substrate diffusion and closed by the bulky heme moiety on the other side. The hydrophobic residues which line one side of the substrate diffusion channel are Pro234, Pro236, Phe380 and Phe381 on one side and Leu421, Phe422, Gln423 and Pro424 on the other side. The substrate binding site consists of the heme
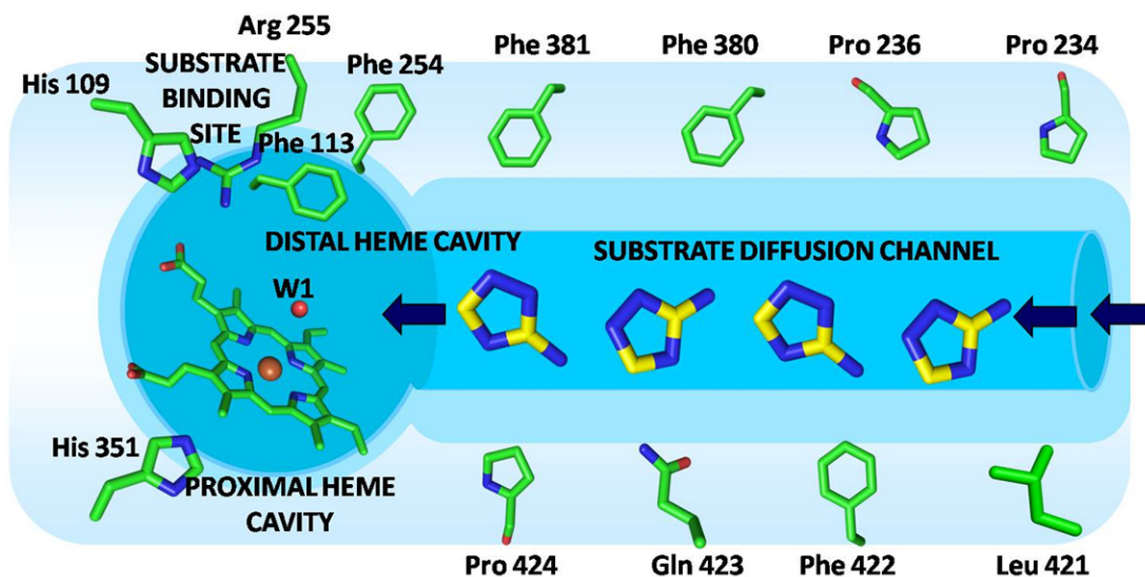
Figure 8. Diagram of the heme cavity of LPO, with the location of distal heme cavity, proximal heme cavity, substrate binding site and the substrate diffusion channel indicated.
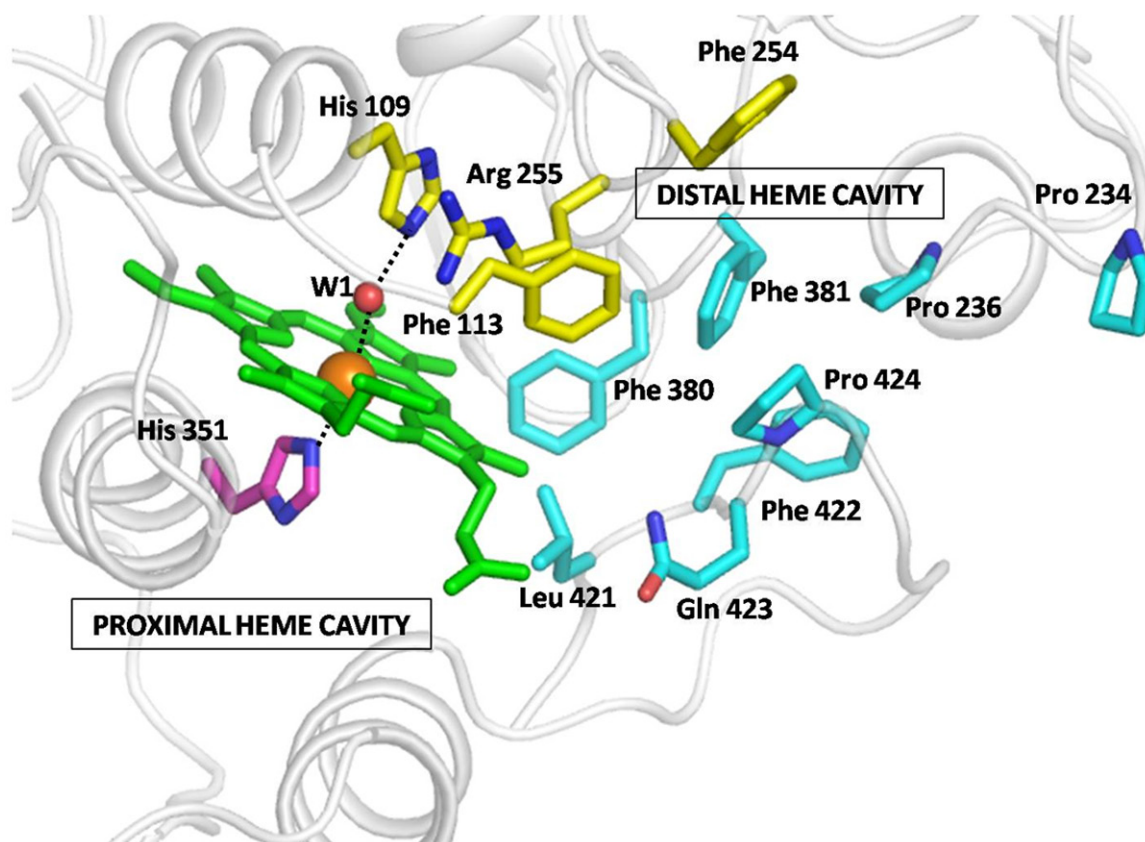

Figure 9. Schematic figure of heme cavity of LPO with the residues of substrate diffusion channel in cyan, the residues of substrate binding site in yellow, heme moiety in green and His351, the only residue in proximal heme cavity in pink.

moiety on one side and residues, His109, Phe113, Phe254 and Arg255 on the opposite side. In between the two sides are two residues, Glu258 to which the heme moiety is
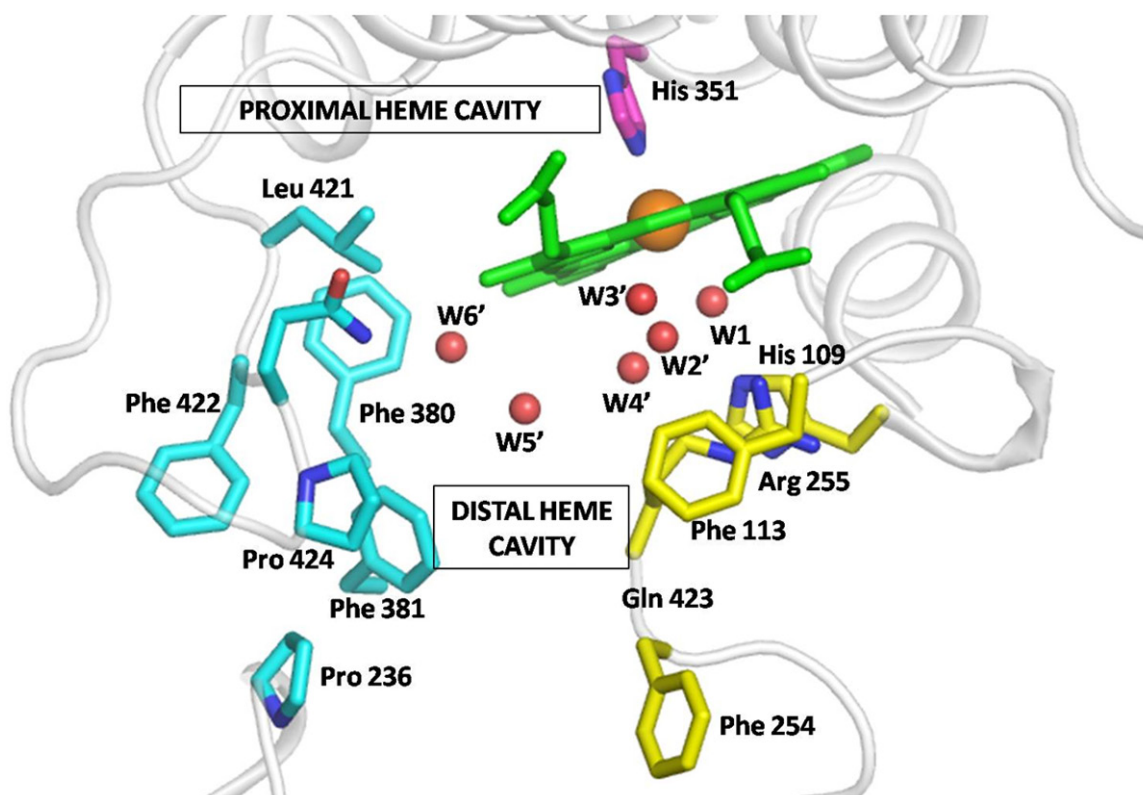
Figure 10. The structure of the substrate-binding site on the distal heme side in LPO and the positions of six conserved water molecules, W1, W2′, W3′, W4′, W5′ and W6′. The proximal and distal sites have been indicated.

covalently bonded, and Gln105 (**Figure 9**). The water network consists of six conserved water molecules, designated as W1, W2′, W3′, W4′, W5' and W6' arranged in a triangular pattern below the heme molecule, with W1 being the closest to the heme (**Figure 10**). In the native molecule of LPO, W1 is connected to the ferric iron of the heme molecule and His109 which functions as a proton donor-acceptor residue. His109 is further connected to five other conserved water molecules, H2, H3, H4, H5 and H6 along with His266 (**Figure 11**).

Substrates and inhibitors of LPO

Though SCN⁻ has been established as the preferred substrate for LPO, there have been several reports in the past which suggest that LPO is capable of binding with various other inorganic ions. Toyama et al reported in 2010 [93] that LPO binds to iodide ion (I⁻) and this binding enhances the catalytic activity of LPO, leading to the conclusion that I⁻ binding has no effect on the optimum pH of activity or the heme structure. This suggested that I⁻ binding in LPO

occurs at a site which is situated away from the heme-linked protonation site.

In another report [94], upon investigation of the binding of several inorganic species like, SCN⁻, I⁻, bromide (Br⁻), chloride (Cl⁻), fluoride (F⁻), nitrite (NO_2^-), cyanide (CN⁻) with bovine LPO using spectroscopic methods, it was found that while all the inorganic ions form 1:1 complexes with LPO, however, SCN⁻, I⁻, Br⁻, and Cl⁻ bind to LPO at a higher affinity as compared to the other compounds. It was also seen that the binding of LPO with SCN⁻ was altered in the presence of other inorganic ions which indicated a competitive binding among these anions.

Though both LPO and MPO are capable of oxidizing inorganic ions, a significant difference between the two is that while the preferred substrate of MPO is Cl⁻, LPO preferentially oxidizes SCN⁻. The two fundamental questions regarding the binding of inorganic ligands to LPO were the identification of the structural parameters that are responsible for the preference of the ions and also, finding the structural basis of the
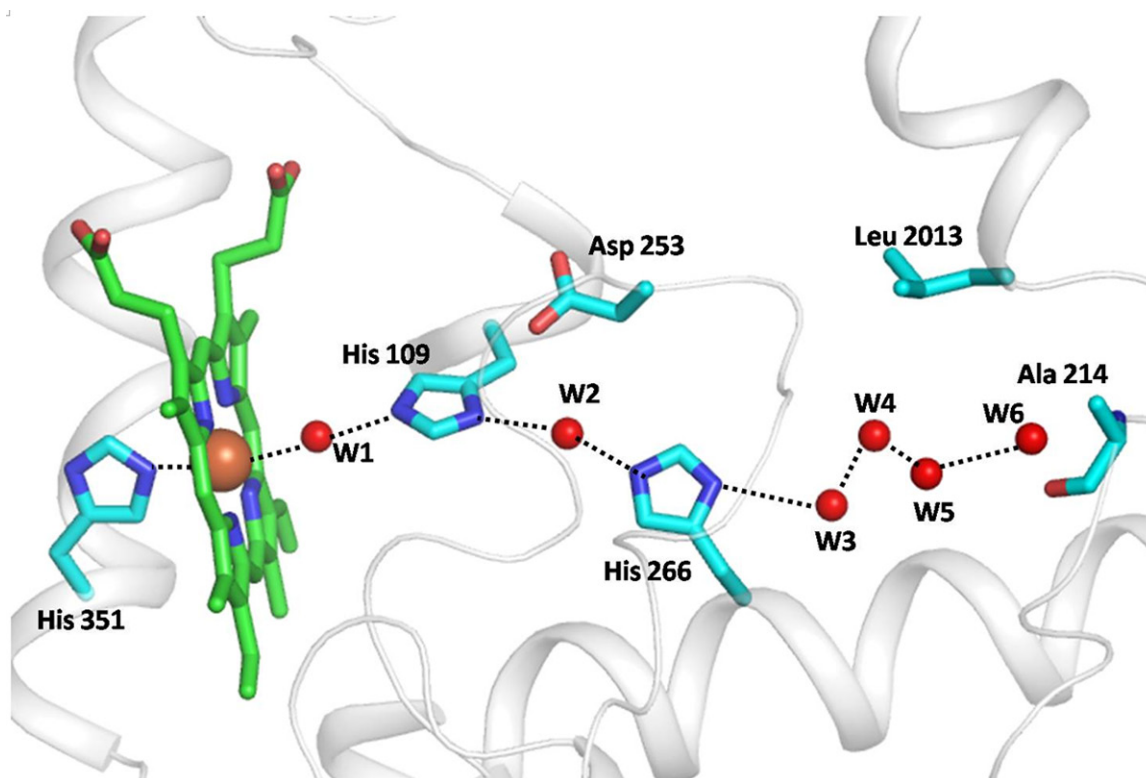
Figure 11. Hydrogen bonded chain involving His351, Fe^{3+}, W1, His109, W2, His266, W3, W4, W5 and W6 where W1 is hydrogen bonded to Fe^{3+} and W6 is located near the protein surface.

preferential binding of SCN^- to LPO as compared to that of MPO.

Structures of complexes of LPO with inorganic substrates

The structures of the complexes of LPO with various inorganic substrates like SCN^- in isolation [42] and with SCN^-, I^-, Br^- and Cl^- [45] have been determined and analyzed in detail. In case of complex of LPO with SCN^- alone, the SCN^- ion was observed to be present in the distal heme cavity, with the sulfur atom of the SCN^- ion in close proximity to the heme iron atom. On the other hand, in the complex of MPO with SCN^- [40], the orientation of the SCN^- is opposite, with the nitrogen atom closer to the heme iron than the sulfur atom. The main reason why SCN^- would be a better substrate for LPO, unlike that of MPO is that the loop Arg418-Phe431 in LPO is found to have a different conformation than the corresponding loop Arg403-Leu415 in MPO, mainly due to the presence of helix H2a in LPO, which is absent in MPO. This leads to varied shapes of the substrate-binding sites and different chemical environments in

case of both proteins. The nitrogen atom of SCN^- in LPO forms a hydrogen bond with a conserved water molecule (W6') which is further bonded to residues Gln423 N 2 and Phe422 O. On the other hand, the corresponding site in MPO is occupied by the bulky side chain of Phe407 in MPO and also, the conserved water (W6') is absent in LPO and hence, it does not generate the same stabilizing interaction for the SCN^- ion. Hence, the stereochemical characteristics of the substrate-binding site in LPO are responsible for the favorable orientation of the SCN^-, as opposed to MPO.

The complex of LPO with inorganic substrates, SCN^-, I^-, Br^- and Cl^- [45] showed that all substrates were bound in the substrate binding site in the distal heme cavity. However, all of them had different positions in the substrate binding site. Water molecules were found in between the substrates (**Figure 12**). A continuous interaction network consisting of W1, Br^-, W5', Cl, W7', W8', SCN^-, W9', I^- was observed in the distal heme cavity of LPO. W1, the conserved water molecule was found in its original position, coordinated to the heme iron on one
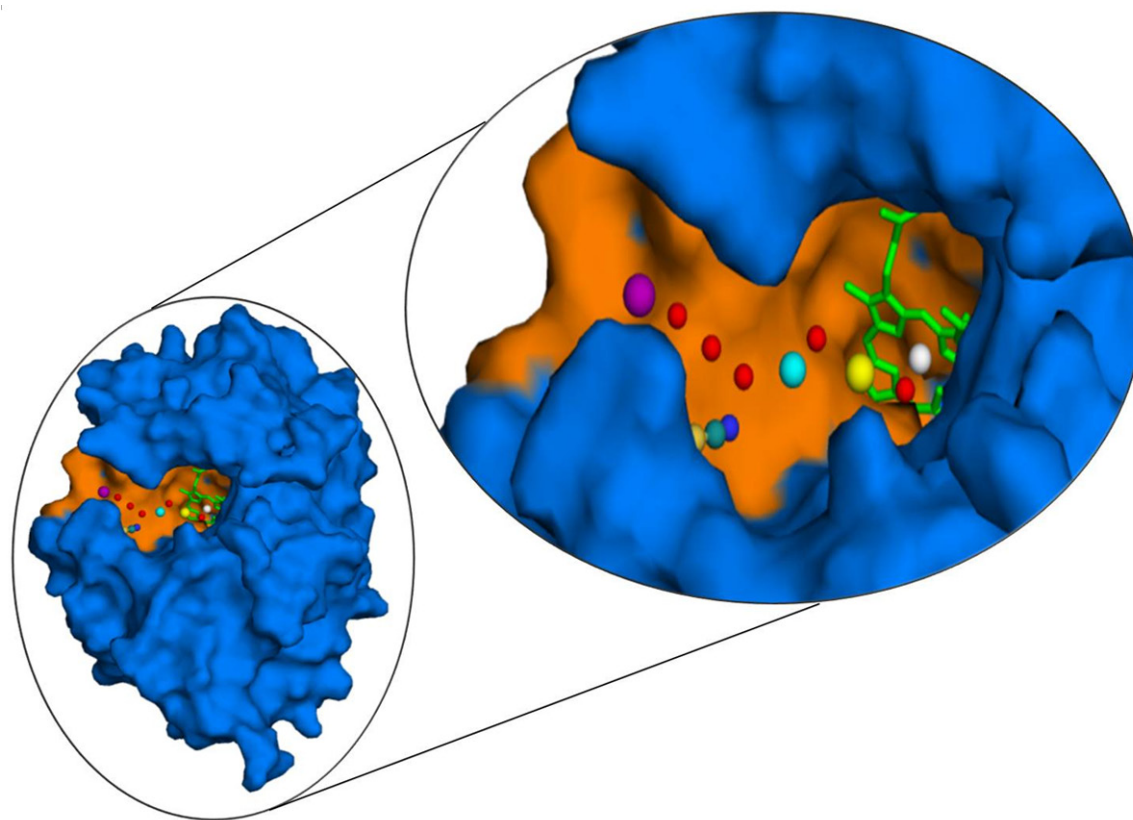
Figure 12. Grasp drawing for the binding of halides with LPO (blue) in the substrate-binding cleft (orange) with heme moiety (green with iron in white), Br⁻ (yellow), Cl⁻ (cyan), I⁻ (purple), SCN⁻ (yellow-green-blue) and water molecules in red.

side and interacting with Br⁻ by a hydrogen bond on the other side. The Br⁻ ion was connected to the by Cl⁻ ion through a hydrogen bonded water molecule W5′ between them. In between Cl⁻ ion and SCN⁻ ion are two water molecules, W7′ and W8′, which are connected through a hydrogen bond network. The SCN⁻ is hydrogen bonded to W9′ which is further connected to the I⁻ ion. Unlike other ions, the SCN⁻ ion makes three more contacts with Asn230 directly and with Ser235 and Phe254 through water molecules respectively (**Figure 13**).

Based on the spatial configuration and the preferred position of each ion, the distal heme cavity was divided into four subsites; S1, containing the heme moiety, W1 and Br⁻ ion, S2 containing W10′ and Cl⁻ ion with W5′ at the junction of S1 and S2, S3 containing SCN⁻, W8′ and W11′ with water molecule, W7′ at the boundary of S2 and S3, and finally, S4 containing I⁻ ion

and W9′. Hence, according to the location of the halides in the substrate binding site, it was concluded that the order of preference of binding of the halides to lactoperoxidase is Br⁻ followed by Cl⁻ which is followed by SCN⁻ and I⁻ at the entry of the channel.

Structures of complexes of LPO with aromatic compounds

Apart from the oxidation of inorganic ions, the proteins of XPO family also catalyze the single electron oxidation of several organic substrates, such as, phenols [95], aromatic amines [96], polychlorinated biphenyls [97], catecholamines, catechols [98-100] and steroid hormones [101-103]. The primary concern regarding the binding of aromatic ligands to LPO was to give the structural basis of the factor which distinguishes between the aromatic ligands in order to make them function as either a substrate or an inhibitor of this enzyme.
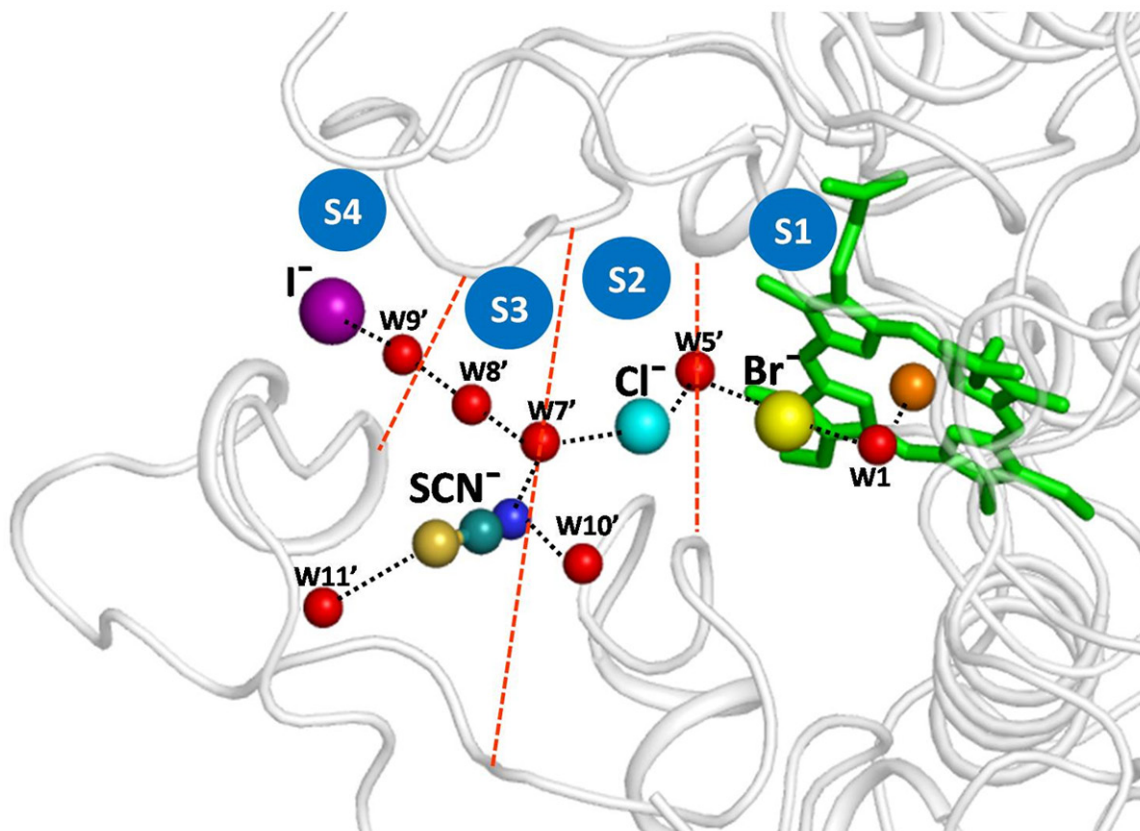
Figure 13. Schematic diagram of LPO structure indicating the binding of four halides, to the substrate binding site on the distal heme side and accordingly, the positions of the four subsites, S1, S2, S3 and S4.

In the past, several aromatic compounds have been reported to inhibit LPO (**Figure 14**). The inhibition of LPO with 3-amino-1,2,4-triazole (amitrole) in the presence of hydrogen peroxide was reported in 1989 [104]. Using NMR spectra, it was demonstrated that amitrole caused a suicide mechanism of inactivation of LPO. In yet another study, the mechanism of suicidal inactivation of LPO by mercaptomethylimidazole (MMI) was studied [105]. It was observed that MMI binds near the iodide-binding site of LPO and inactivates the enzyme. Similarly, resorcinol was also demonstrated to cause an irreversible loss of enzymatic activity substantiated by kinetics which suggested a suicide mechanism [106]. The binding of resorcinol caused a drastic change in the visible spectrum of the prosthetic heme group. Interestingly, the inactivation of resorcinol was specific only for LPO and TPO but did not show any inactivation in case of MPO.

In yet another study, salicylhydroxamic (SHA) and benzohydroxamic acids (BHA) were demon-

strated to be inhibitors of MPO and play a significant role in binding of the ligand to the heme iron [107]. The results clearly indicated that the hydroxamic side chains of SHA and BHA interact directly with the heme iron. Similar results on LPO suggested that the same aromatic ligands would bind and inhibit LPO as well. In yet another related report, aromatic substrates such as phenol, p-cresol, resorcinol, and 4-amino salicylate were found to be bound to an aromatic binding site near the heme center of MPO using EPR spectroscopy and model building [108].

Ozdemir et al reported the non-competitive inhibition of LPO by two drugs, Ketamine and Bupivacaine in 2005 [109]. However, another drug, propofol, which is used as a hypnotic intravenous agent, was found to be a competitive inhibitor of LPO [110]. Since then, a number of studies on aromatic inhibitors of LPO have been reported, for example, sulphanilimide [111] and melatonin and serotonin [112]. In yet another study, mono- and disaccharides
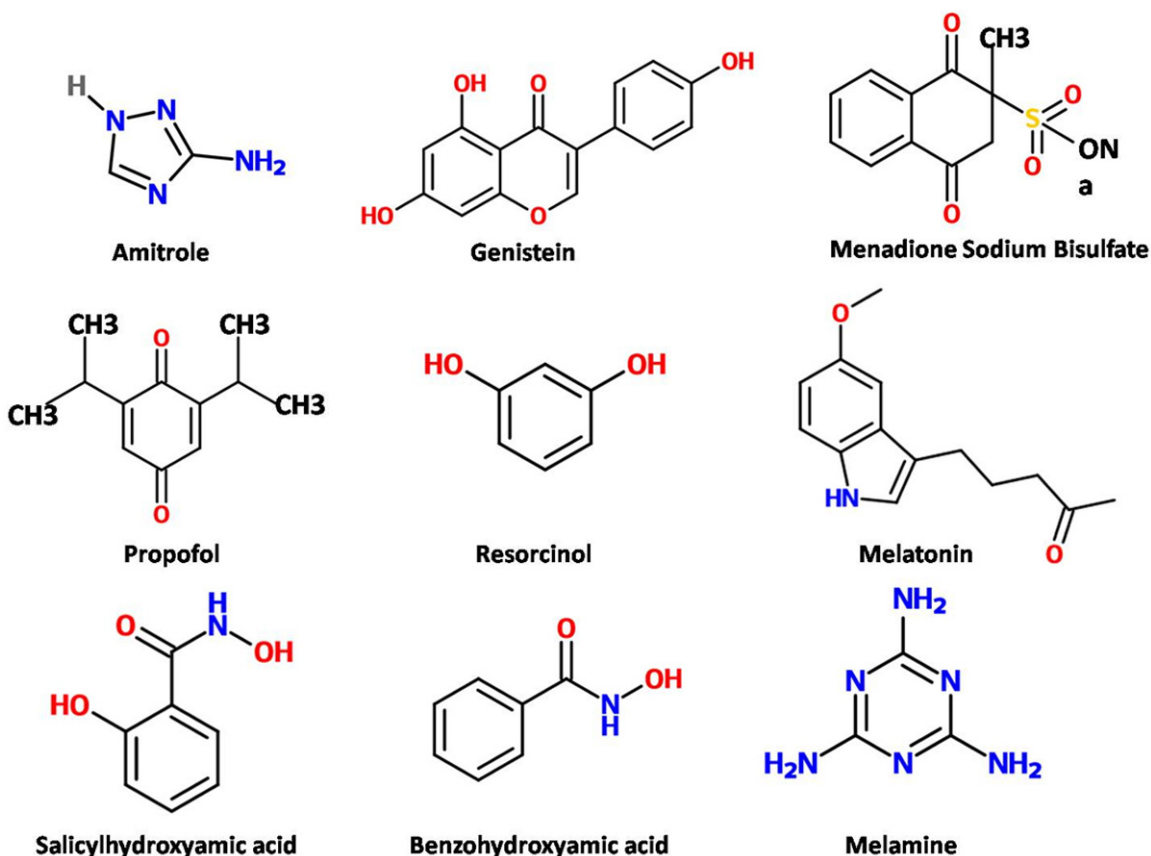
Figure 14. Structures of some of the reported aromatic compounds which act as inhibitors of LPO.
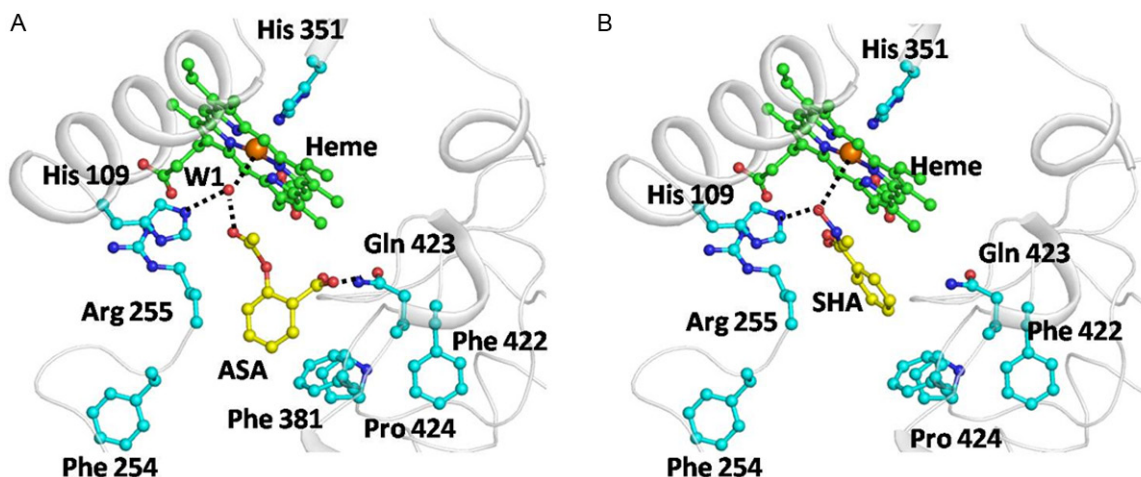

Figure 15. A: Acetyl salicylic acid (ASA) bound to LPO as a substrate at the substrate-binding site (B) Salicylhydroxamic acid (SHA) bound to LPO as an inhibitor in the substrate-binding site. The hydrogen bonded interactions are indicated by dotted lines.

were found to inhibit LPO [113]. Similarly, vitamins such as L-ascorbic acid (Vitamin C), menadione sodium bisulfate (vitamin K3), and folic acid were also found to be potent competitive

inhibitors of LPO [114]. Genistein, a soy isoflavone metabolite was also found to inactivate LPO by irreversible covalent binding but there was no evidence of any interaction of genistein

interaction with the prosthetic heme moiety [115]. Recently, melamine, an organic base with a 1,3,5-triazine skeleton was shown to inhibit LPO in a non-competitive way [116].

The structural evidence for the inhibition of LPO by aromatic compounds was observed by analyzing the structures of complexes of LPO with acetylsalicylic acid (ASA), SHA and BHA [41] and with amitrole [45]. From the analysis of the crystal structures, it was evident that all the compounds bound the LPO molecule in the substrate binding site in the distal heme cavity. However, there was one remarkable and consistent difference found in the binding of inhibitors vs. substrates to LPO. It was observed that though all substrates bind to LPO, this binding does not lead to dislocation of conserved water molecule, W1. However, the binding of inhibitors to LPO leads to the expulsion of W1 from the substrate diffusion channel. Hence, ASA, which is a known substrate of LPO, bound to the distal heme cavity without disturbing the conserved water molecule W1. In fact, the acetyl group of ASA interacts with the heme iron through an intermediate hydrogen bond with W1 (**Figure 15**). On the other hand, SHA, BHA and amitrole, which are inhibitors of LPO, bound to LPO in such a manner so that W1 was replaced by the hydroxyl (OH) group of their hydroxamic acid moieties. Since the W1 is absent in their cases, OH group of hydroxamic acid moiety mimics the interactions of W1, and thereby inhibits LPO.

Conclusions

LPO is a potent antimicrobial protein found in milk which belongs to the family of mammalian heme peroxidases. Though it had been studied in detail over the last thirty years, its three-dimensional structure has been elucidated recently. Though its structure has been found similar to that of MPO, there are significant differences between the two proteins. The structural basis of LPO's preference of certain substrates over the other has been understood. Also, the mechanism of inhibition of LPO by several aromatic ligands has been discovered by analysis of complexes of LPO with known inhibitors. A pertinent question related to why a certain aromatic ligand acts as an inhibitor or substrate has been answered. More studies need to done on this fascinating molecule in order to completely understand the mechanisms of its action so that it could be used in medicine and industry more effectively.

Disclosure of conflict of interest

None.

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