INTRODUCTION

Studies on metallo proteins have attracted widespread attention owing to the crucial roles of the metal ions in various biological functions. The importance of the metal ions is clear from the fact that about one-third of the known proteins require metal ions for their functions. Metal ions are involved in a multitude of biological processes. Many critical processes such as respiration, nitrogen fixation, photosynthesis, regulation of the synthesis of ribonucleic acid (RNA) and protein, protection against toxic and mutagenic agents as well as processes such as nerve transmission, muscle contraction, signal transduction require metal ions [1,2,3,]. Diverse functions played by metal ions in biological systems are influenced by the molecular structure inclusive of ligand environment as well as their redox and ionic states. Most metal ions are essential as trace elements for the biological function of living organisms. Some of the important among these are: Na+, K+, Mg2+, Ca2+ and the transition metal ions such as V, Mn, Fe, Co, Ni, Cu, Zn, and Mo.

Iron is the most important essential trace element in living organisms; 45–55 mg of iron per kg of body is found in an average human being [4]. Nature widely exploits iron’s ability to co-ordinate electron donors and to participate in redox processes to perform a variety of physiological functions under very mild conditions present inside the cell. Iron containing proteins can broadly be divided into two different classes:

(i) heme proteins and (ii) non heme proteins. Heme proteins are an important class of metalloproteins which are involved in various biological functions [1,2]. Presence of iron porphyrin complex, (protoporphyrin or modified protoporphyrin) as a prosthetic group in the active site is a common feature of all these proteins (Fig. 1.1). The prosthetic group is attached to the protein via fifth co-ordination site of iron and the sixth position is either vacant where different substrates / ligands can bind or co-
Figure 1.1: Iron III complex of protoporphyrin IX (heme) (A); Iron III complex of 8-mercaptomethyl protoporphyrin IX (prosthetic group of lactoperoxidase by Nichol, et al (1987).
ordinated to protein via another amino acid residue depending upon functional requirement of the protein. The nature of axial ligands, the redox potentials, spin oxidation states of iron and protein environment around the active site play crucial role in controlling the reactivity of the heme iron. The diversity of different heme proteins are controlled by two factors:

(a) The geometry and the nature of ligands attached to the heme. For example in hemoglobin (Hb) and myoglobin (Mb), the iron of the heme is bonded to histidine on one side, other side is used to bind oxygen for transport and storage. Cytochrome c has the heme C unit with two axial ligands, a histidine and a methionine, and functions as an efficient electron carrier. Cytochrome P 450 and chloroperoxidase both have identical thiolate group as axial ligand, but cytochrome P 450 functions as monoxygenase, and chloroperoxidase functions as a halogenate. (b) The protein environment around heme prosthetic group, including the polarity of the immediate surrounding and steric constrains on the accessibility of substrates.

A wide variety of heme proteins is found in nature, which use \( \text{H}_2\text{O}_2 \) to catalytically oxidize substrates or to incorporate oxygen atoms into organic substrates. Enzymes which catalyze the oxidation of various organic and inorganic compounds by \( \text{H}_2\text{O}_2 \) or related compounds are called peroxidases [5]. Lactoperoxidase (LPO, EC 1.11.1.7), a mammalian peroxidase, is a product of secretion of exocrine glands responsible for secretion of saliva and tears [6-8]. The protein part of LPO consists of a single polypeptide chain of 612 amino acids, and a heme as prosthetic group. The enzyme has a molecular weight of 78,500. Its normal physiological role is as a bactericidal. The bacteriocid function of LPO is also of primary importance for the closely related salivary peroxidase (SPO) and myleoper-oxidase (MPO). LPO has recently been found in human colostrum and can be a useful criterion for distinguishing between hormone – dependant and hormone – independent types of mammary cancer [9]. The antibacterial activity of bovine milk was recognized at about the same time as that of blood and Hanssen [10], was first to suggest that the bacterial activity of raw bovine milk against \( \text{Bacillus typhosa} \) and \( \text{B. paratyphosa} \) was associated with the oxidases and peroxidases occurring in milk. The inhibition occurred only under aerobic conditions and could be reversed by reducing agents [11,12]. Another functional role for LPO is in the degradation of catecholamines such as norepinephrine [13]. Soluble
LPO is both a functional and a spectral model for membrane-bound thyroid peroxidase (TPO) [8,14].

<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
<th>Isolated from</th>
<th>Mol. Wt.</th>
<th>Prosthetic group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plant Peroxidase</strong></td>
<td>Horseradish peroxidase</td>
<td>Root of horse-radish plant</td>
<td>40,000</td>
<td>Ferriprotopor-phyrin IX</td>
</tr>
<tr>
<td></td>
<td>Turnip peroxidase</td>
<td>Turnip plant roots</td>
<td>45,000</td>
<td>-do–</td>
</tr>
<tr>
<td></td>
<td>Japanese radish peroxidase</td>
<td>Roots of Japanese radish plant</td>
<td>50,000</td>
<td>-do–</td>
</tr>
<tr>
<td><strong>Microorganism peroxidase</strong></td>
<td>Cytochrome C peroxidase</td>
<td>Baker’s yeast</td>
<td>34,100</td>
<td>-do–</td>
</tr>
<tr>
<td></td>
<td>Thyroid peroxidase</td>
<td>Hog Thyroid glands</td>
<td>62,000</td>
<td>?</td>
</tr>
<tr>
<td><strong>Mammalian peroxidase</strong></td>
<td>Lactoperoxidase</td>
<td>Cow’s milk</td>
<td>78,500</td>
<td>8-Mercapto-methyl derivative of Ferri-protoporphyrin IX having ester groups at positions -1 &amp; -5, and vinyl moieties at -2 &amp; -4 positions.</td>
</tr>
</tbody>
</table>

Lactoperoxidase is the most abundant enzyme in bovine milk, constituting about 1% of the whey proteins (total protein less casein) or 10 – 30 µg/ml of milk. LPO occurs in the whey of all cow’s milk, varying slightly between individuals and possibly breeds. Lactoperoxidase and salivary peroxidase, a closely related peroxidase in primary structure and properties to LPO, catalyze the oxidation of the thiocyanate and iodide ions to generate highly reactive oxidizing agents. LPO in combination with H₂O₂ and thiocyanate (SCN⁻) shows antibacterial activity and forms...
an important component of the biological defense system present in mammals. These products have a broad spectrum of antimicrobial effects. They can kill viruses, gram – positive and gram – negative bacteria, fungi, and probably also mycoplasmas and parasites. The molecular components of cells that are oxidized are sulfhydryl groups, NADH, NADPH and under some conditions aromatic amino acid residues. Oxidation of these molecular components alters the functions of cellular systems [6]. The relationship of lactoperoxidase to other mammalian peroxidases such as chloroperoxidase (CPO), thyroid peroxidase (TPO), myeloperoxidase (MPO), eosinophilic peroxidase (EPO), intestinal peroxidase (IPO) and salivary peroxidase is of interest.

The series of mammalian peroxidases, LPO, SPO, TPO, eosinophil peroxidase, intestinal peroxidase and myeloperoxidase (MPO), have been found to have related protein primary structures [15,16] and prosthetic heme moieties which are not readily extracted by conventional approaches [6-8]. The proteins also show spectral features that are distinct from those of known heme groups [6-8], as a consequence, the structural identity of the heme moieties of mammalian peroxidases is still not very clear. Clezy and colleagues [17], proposed the LPO heme structure (Fig. 1.2) by treatment of LPO with 2-mercaptoethanol and urea to gave 8-thiomethylene-substituted derivative of iron protoporphyrin IX (Fig.1.2B). This hypothesis explained the lack of ready extractibility for the LPO heme moiety but does not fully explain about the spectral variations from heme. Heme (iron III protoporphyrin IX) (Fig. 1.1) is present in many common heme proteins as in myoglobin (Mb), hemoglobin (Hb), the P450 enzymes, catalases and peroxidases from yeast, plant and fungal sources i.e., cytochrome c peroxidase, horseradish peroxidase, lignin peroxidase and chloroperoxidase. Sievers [18] proposed that the anomalous electronic absorption spectrum of LPO and its pyridine hemochrome derivative, relative to those of known heme containing proteins, probably arose from an unusually constrained active-site pocket surrounding the heme. Most recently, Fenna and colleagues [16] and Taylor et al [19], have determined the structure called “heme m” prosthetic group of native MPO (Fig.1.2C). Fenna et al [16] suggested that close similarities in the protein sequences of MPO and LPO made it possible that LPO could have covalent heme-
Figure 1.2: Heme structure of lactoperoxidase. (A): Iron protoporphyrin IX (heme b). (B): 8-thiomethylene-substituted heme b. (C): the m heme of myeloperoxidase.
protein linkages and a heme structure related to that of MPO. Evidence for the identity of the LPO heme has come from a variety of different experimental approaches. These include resonance Raman \[20,21\], NMR \[22,23\] and EPR \[24\] spectroscopy etc. Andersson, et al \[25\] data demonstrated obvious spectral differences between the hemes of LPO and Mb or HRP, and between the hemes of LPO and MPO, in both native and \Fe^{3+} - \text{CN}^-\ forms. Furthermore, the spectral differences were not derived from axial ligands, as shown by comparison of native LPO and Mb -formate. Thus Andersson \[25\] suggested that the LPO heme (called heme 1) is distinct from either heme \(\alpha\) or heme \(m\), although it has similar peripheral substituents to each. With the study of high degree of spectral and protein sequence agreement between mammalian peroxidases \[15,16,19\], he suggested that the heme 1 of LPO, or a very closely related heme, is likely also be the heme of TPO, SPO, eosinophil peroxidase, and other mammalian peroxidases. The proposed structure of the prosthetic group of LPO as shown by Andersson, et al \[25\] is given in (Fig 1.3). They suggested that the LPO heme moiety, which he called heme 1, has vinyl moieties at the 2- or 4- positions as in the case of heme \(b\). In addition, the heme 1 of LPO has ester groups at positions 1 and 5 like heme \(m\) of MPO.

Important factors with respect to the heme of native LPO are (1) the nature and identity of the 5th axial ligand to the heme moiety and (2) the presence or absence of 6th ligand. The 5th ligand, donated by the protein, is known to have a considerable effect on the observed spectral properties as well as on functional characteristics. The 5th ligand of heme of LPO is either a histidine \[28\] or the deprotonated N-His \(\text{~}\) common to many peroxidases \[22,24,26,27\]. The 6th ligand of LPO has variously been reported to be an unusual formate \[28\] or \(\text{H}_2\text{O}\) \[20,21\]. Some assignment in consistencies exist such as a reported 5–co-ordinate structure \[27,29\], whereas the body of other evidence indicates that native LPO is 6–co-ordinate \[20,21,30\]. Andersson, et al \[25\] addressed the heme moiety of LPO with respect to the structural proposals in Figure 1.2. The approach was comparative spectral analysis of LPO and the analogous forms of: (1) Mb and HRP, both of which have heme \(b\) and respectively either N–His or N–His 5th ligands; and (2) MPO which has the novel heme \(m\) and N–His ligation \[16,19\]. MCD spectroscopy
Figure 1.3: The structure proposal for the heme 1 moiety of lactoperoxidase [Andersson, et al, 1996].
was able to distinguish easily between 5- and 6-co-ordinate ferric heme systems [31-33] and is sensitive to heme bound water [31,33].

A theoretical three-dimensional model for lactoperoxidase and eosinophil peroxidase, built on the scaffold of the myeloperoxidase x-ray structure was recently suggested by Rosa Pia Ferrari, et al (1996) [34] (Fig. 1.4). The three mammalian heme containing enzymes, myeloperoxidase (MPO) (EC 1.11.1.7), eosinophil peroxidase (EPO) (EC 1.11.1.7) and LPO (EC 1.11.1.7), found in body fluids and tissues are among the most studied members of the mammalian peroxidase family. MPO is the only member of this family for which the x-ray derived structure is presently available [35,36]; for bovine LPO [37,38] and human EPO [39], only primary sequences are known. The hemoproteins show highly α-helical structures [40], and MPO follows the same trend. It was suggested that almost all the regions corresponding to helices in MPO are well conserved in LPO and EPO. The only exception was given by the amino acid stretch extending from residue 470 to residue 475 of the MPO sequence, which was not conserved in the other two proteins. LPO shares some common features with MPO [41,42], and several suppositions have been made about the nature of its heme–protein interaction. The conclusion given by Ferrari, et al were: (1) The theoretical calculations account for the presence of ester bonds linking the heme group to the apo proteins of LPO and EPO, in agreement with the experimental results of both Hultquist and Morrison [43] and Rae and Goff [44]; they, however, do not support the hypothesis of Nichols and colleagues [45] about the existence of a disulphide linkage between heme and LPO. (2) The folding and primary sequences of the proteins are strictly correlated in order to ensure a well conserved heme environment. (3) The localization of a hydrophobic access channel on the protein distal site matches the reactivity with aromatic substrates.

The crystal structure of horseradish peroxidase (HRP, EC 1.11.1.7, donor–H$_2$O$_2$ oxireductase) has recently been reported [47]. Primary structure contains 368 amino acids [46]. The protein part is attached through the nitrogen atom of the imidazole side chain of histidine (His$^{170}$) to the iron of protoporphyrin IX (heme b). The proximal histidine–iron protoporphyrin bond of HRP is labile and can be cleaved at low pH. This property has been exploited in reconstituting HRP with various metal...
Figure 1.4: Schematic drawing of polypeptide backbone of bovine lactoperoxidase. The theoretical model of the three dimensional structure developed on the basis of x-ray structure of MPO by Gioia et al, 1996, [34], showing relative orientation of heme moiety and the tryptophans.
substituted porphyrins in order to study the effect of central metal substitution on the peroxidase activity of HRP. There are also eight neutral carbohydrate side chains attached through Asn residues 13, 57, 158, 186, 198, 214, 250 and 268, four disulphide bridges between cystein residues 11-91, 44-49, 97-301 & 177-209 and two Ca$^{2+}$ ions present per HRP molecule. The total molecular weight of HRP is around 42,100.

The recent crystal structure of horseradish peroxidase isoenzyme C (HRP C) by A. T. Smith ; et al, (1997) [47] essentially confirms the mechanism of peroxidase action that has become well known. The mechanism involves the ferric enzyme which reacts rapidly with hydrogen peroxide to form compound I [48], (Fig. 1.5) an oxyferryl species in which one electron had been withdrawn from the heme group to form a porphyrin π cation radical. This intermediate is reduced in two sequential one electron steps through compound II (in which the porphyrin cation radical has been reduced):

\[ (a) \quad E(\text{Fe}^{3+})\text{Porp} + \text{H}_2\text{O}_2 \rightarrow E(\text{Fe}^{4+}=\text{O})\text{Porp}^{**} + \text{H}_2\text{O} \quad (1) \]

\[ \text{Compound I} \]

\[ (b) \quad E(\text{Fe}^{4+}=\text{O})\text{Porp}^{**} + \text{AH} \rightarrow E(\text{Fe}^{4+}=\text{O})\text{Porp} + \text{A}^* \quad (2) \]

\[ \text{Compound II} \]

\[ (c) \quad E(\text{Fe}^{4+}=\text{O})\text{Porp} + \text{AH} \rightarrow E(\text{Fe}^{3+})\text{Porp} + \text{A}^* + \text{H}_2\text{O} \quad (3) \]

Where \( E = \text{HRP} / \text{or LPO} \)

The central feature of mechanism is the formation of spectroscopically distinct intermediate, compound I, by the action of oxidizing agent on the native enzyme. Compound I of HRP which has been by far the most extensively studied, was detected as a very short lived green intermediate by Theorell [59], although relatively more stable red intermediate, compound II was detected earlier [2]. Several lines of intermediate have demonstrated that: (i) Compound – I is formed by the action of \( \text{H}_2\text{O}_2 \) on HRP in 1:1 stoichiometry [61]. (ii) It is two oxidizing equivalents above
Figure 1.5: The catalytic cycle of Peroxidases. The overall charge on peroxidase (1) and compound I (2) is +1, whereas, compound II (3) is neutral.
native enzyme [62,63]. (iii) HRP–I is in oxo ferryl state (Fe^{IV}=O) with one electron removed from the porphyrin producing radical cation species [64,65]. (iv) Compound –II is obtained by one electron oxidation of the substrate. It is also present in oxy – ferryl (Fe^{IV}=O) form carrying only one oxidizing equivalent above the native enzyme [63,66]. Fig.1.5 shows the catalytic cycle of the oxidation of substrate by peroxidases.

The high rate constants and low activation energies ($\leq 4k \text{ cal mol}^{-1}$) for pH independent reactions between active enzyme and H$_2$O$_2$ have led to the suggestions that the reactions may be diffusion controlled [67], but a number of lines of evidence indicated [68,69] that they are chemically controlled and involve reversible formation of precursor complex HRP-H$_2$O$_2$ where it is implied that no electron transfer has occurred between the reactants.

Recent low temperature stopped flow experiments have shown the formation of compound 0, which is also a precursor complex and has unusual optical spectrum [69].

(II) \[ \text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{HRP - H}_2\text{O}_2 \rightarrow \text{Compound 0} \rightarrow \text{Compound I} \]

$$\quad \qquad (k_1, k_{-1}) \quad \quad (k_2, k_{-2})$$

Compounds 0, I and II occur as intermediates in the enzyme function of peroxidases.

Type of mechanism that operates in a given reaction depends on the nature of enzyme, substrate as well as their state of protonation. Oxidation of organic substrates such as phenols and aromatic amines generally proceeds according to equations 1 to 3 [70].

Oxidation of inorganic anions such as iodide, bisulphite, sulphite and thiocyanate ions generally proceed by two electron path way. Acidic to neutral pH generally have been found to favor two electron path way. Oxidation of sulphite catalyzed by HRP has been shown to change mechanism from two electron path way to one electron path way as pH is increased [71]. Nitrite anion on the other hand has been shown to follow usual one electron oxidation path way [72,73]. The one electron and two electron
mechanisms in enzymatic oxidation – reduction reactions have been discussed in detail [74,75]. Evidence for oxygen atom transfer from H₂O₂ or solvent water has been reported recently in the LPO / HRP catalyzed oxidation of organic sulphides [76,77]. A two step oxygen transfer mechanism involving a cation radical intermediate has been suggested from the steady state kinetic studies, which has also been confirmed by transient state kinetic studies [78].

The role of protein in catalytic action:

The protein moiety in heme proteins does not merely provide a hydrophobic container to solubilize the hydrophobic heme group. The interactions that hold the heme group in protein matrix include : (a) Van der waals interactions between hydrophobic side chains and the heme π system. (b) Co-ordination of central iron atom to proximal residue which is histidyl imidazole in CCP, HRP and LPO, but thiol ligand in CLP and (c) The electrostatic interactions between the residues in the active site heme.

In lactoperoxidase (LPO), distal histidine has been suggested to play important role in mediating the electron transfer from the oxidizable substrate to iron, forming a route

\[ S-H^+ -N-C-N-H- \ldots O = Fe^{IV} \] \[ 84. \]

\( S \) is oxidizable substrate. Since Fe⁴⁺ =O of compound II associates with the NH of the imidazole ring of distal histidine by hydrogen bonding [85].

Nature of substrate binding

Mechanisms of substrate oxidation's by compound I or compound II (Fig. 1.4), involve binding oxidisable substrate to the native peroxidase at appropriate site [86-88]. Existence of definite binding site for aromatic donors on HRP and LPO was shown [89]. Paul and Ohlsson [90] also suggested that the binding site for aromatic donors on HRP is likely to be by a hydrogen bonding and hydrophobic interaction in the vicinity of the aromatic amino acid residue. A hypothetical model of the enzyme
structure of HRP obtained by computer aided simulation procedures suggest that binding of these donor molecules occur in the vicinity of the heme peripheral 8-methyl group with hydrophobic interactions probably with Tyr 185 and with hydrogen bond with adjacent amino acid residues such as Arg 183 [91]. Essentially the same structure of the active site is predicted from the reaction of HRP with phenyl hydrazine [92] and alkyl hydrazines [93]. Phenyl hydrazine produces partially in attachment of phenyl group to the 8-meso carbon and partially in conversion of 8 hydroxy methyl derivative, while reaction with alkyl hydrazine produce derivatives in which same 8-meso carbon gets alkylated. This was interpreted as the only sector of heme perimeter defined by the edge of pyrrole ring D and 8-meso carbon is physically accessible to substrates.

We have characterized lactoperoxidase by steady-state intrinsic fluorescence quenching studies. Effect of denaturing agents like GdnHCl on the fluorescence of lactoperoxidase was also examined.

Circular Dichroism (CD) spectroscopic studies on LPO were also carried out by us. The backbone circular dichroism spectra of LPO in presence of urea and guanidine hydrochloride showed that LPO is as conformationally stable as plant peroxidase HRP-C.

In the present thesis, we have characterized bovine lactoperoxidase using steady-state fluorescence spectroscopy. Intrinsic fluorescence of LPO and its ligand bound derivatives were studied. Tryptophan fluorescence changes occurring during denaturation of LPO in presence of Guanidine hydrochloride were studied and these results were compared with CD spectra of the back bone. Quenching of tryptophan fluorescence by iodide, cesium ion and acrylamide were studied (chapter 3). We have measured the second order rate constants of one electron oxidation of some para-substituted phenols by the enzyme intermediates, HRP-II and LPO-II. We have compared them with the redox potentials of the substrates. Reorganization energy of electron - transfer was estimated from the variation of their second order rate constants with the thermodynamic driving force. Interaction of heme and synthetic heme with heme carrier protein bovine serum albumin have been studied using fluorescence quenching technique. Heme protein association constants were estimated and nature of affinity have been discussed.
Table 1.2: Amino acid analysis of LPO and HRP

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>HRP</th>
<th>LPO-B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>6</td>
<td>34</td>
</tr>
<tr>
<td>Histidine</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Arginine</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>46</td>
<td>71</td>
</tr>
<tr>
<td>Theonine</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td>Serine</td>
<td>26</td>
<td>33</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>20</td>
<td>61</td>
</tr>
<tr>
<td>Proline</td>
<td>17</td>
<td>42</td>
</tr>
<tr>
<td>Glycine</td>
<td>17</td>
<td>40</td>
</tr>
<tr>
<td>Alanine</td>
<td>23</td>
<td>37</td>
</tr>
<tr>
<td>½ cysteine</td>
<td>8</td>
<td>16e</td>
</tr>
<tr>
<td>Valine</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>Methionine</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td>Leucine</td>
<td>35</td>
<td>68</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1</td>
<td>15</td>
</tr>
</tbody>
</table>
REFERENCES

University Science Books, Mill Valley, CA.

*Bioinorganic Chemistry*, University Science Books, Mill Valley, CA.


*Biochim, Biophys. Acta* 742, 659-668.


261, 6734-6741.

[31] Ikeda-Saito, M., Hori, H., Andersson, L. A., Prince, R. C., Pickering, I. J.,

George, G. N., Sanders, C. II, Lutz, R. S., McKelvey, E. J., and Mattera, R.,


*FEBS Lett.* 370, 97-100.

[34] Gioia, De Luca., Ghibaudi, M. E., Laurenti, E., Salmona, M., Ferrari, P. R.,


[37] Dull, T. J., Uyeda, C., Strosberg, A. D., Nedwin, G., Seilhamer, J. J., (1990),

*DNA Cell Biol.* 9, 499-509.


198, 733-739.


