CHAPTER 3

CHARACTERIZATION OF BOVINE LACTOPEROXIDASE:
CIRCULAR DICHROISM AND TRYPTOPHAN FLUORESCENCE
QUENCHING STUDIES:

3.1 Introduction:

Heme peroxidases are a class of enzymes that efficiently catalyse oxidation of large number of organic and inorganic compounds using hydrogen peroxide or hydroperoxides as oxidising agents [1,3]. Lactoeroxidase (LPO)(donor:hydrogen peroxide oxidore-ductase, (EC 1.11-1.7), a redox enzyme with antibacterial properties, found in several biological fluids like milk, tears, saliva, etc, is a member of mammalian peroxidase family. It has an important function in biological defence mechanisms [3,4]. LPO catalyses oxidation of thiocyanate and iodide ions to generate highly oxidising and toxic products which can kill viruses, gram positive and gram negative bacteria, fungi, parasites, tumour cells and also mycoplasmas [5].

Deoxyribose nucleic acid (DNA) and primary sequence of (bLPO) major cationic form, previously isolated by Calstrom [11] have been determined. bLPO consists of a single polypeptide chain of 612 amino acids [12,14,15], molecular mass of 78,500 Da, a heme and about 10% carbohydrate content [11,15,10,16]. There are 15 tyrosines and 15 tryptophan residues in the enzyme [14,15,23]. The identity of the covalently bound heme group (protoheme) has been characterised in several studies by UV-Vis [16,11], CD [13], NMR[18,6,7,19], EPR [16,20,21], Resonance Raman [24], EXAFS[25], and MCD[22]. The protoheme in bLPO was suggested to be bound to the peptide chain via a disulphide linkage [17,12]. Recent experimental results by Rae and Goff [19] and by Anderson et al [22] suggested, however, that the bLPO heme is linked to the peptide by one or two ester bonds to glutamate and aspartate residues. X-ray structure for bLPO is not available, however, recently a theoretical three dimensional model, built on the scaffold of MPO x-ray structure has been proposed for bLPO [23].
Several studies have been reported on bLPO [26,27], however, very little is known about its stability and the nearest surroundings of the protoheme. It was suggested that tryptophan in bLPO are not essential for the enzymatic activity [31,32], however, intrinsic tryptophan fluorescence offers a measure of the protein conformational state, independent of the spectroscopic properties of the heme group. Circular dichroism has also proved to be useful tool in elucidating the structure of proteins [28]. In this work, the intrinsic fluorescence of bLPO and its ligand bound derivatives were studied. Tryptophan fluorescence changes occurring during denaturation of bLPO in presence of GdnHCl were studied and these results were compared with CD spectra of the back bone. Quenching of tryptophan fluorescence by I-, Cs+, and acrylamide were studied.

3.2 Materials & Methods

N-acetyl-L-tryptophanamide (NATA, AcTrpNH2), acrylamide, urea, and guanidine hydrochloride (GdnHCl) were obtained from Sigma. Analytical grade KI and NaCl were obtained from Glaxo, India Ltd. and used without further crystallisation.

bLPO was isolated and purified from 30 litres of unpasturized, raw cows milk, essentially by the procedure of Dumontet and Rousset [33]. The crude bLPO was extracted by stirring milk for three hours with ion exchanger CG-50-NH4. It was eluted by 0.5 M sodium acetate. After precipitation by ammonium sulphate (NH4)2 SO4, and extensive dialysis against 5mM sodium phosphate buffer, pH 6.8, the final purification of bLPO was carried out by ion exchange chromatography (×2) on CM-sepharose-CL-6B using 5mM–200mM sodium phosphate buffer gradient at pH 6.8. The final bLPO fractions with Rf A412 / A280 = 0.94 were pooled together.

3.3 Spectroscopic Measurements:

Absorption spectra were recorded on Shimadzu UV-2000 spectrophotometer. The fluorescence quantum yields for bLPO and its derivatives in buffer relative to the tryptophan standard, AcTrpNH2, were determined from the ratios of the areas of the emission bands normalised for the relevant absorption at the excitation wave length
(295 nm). Ionic strength of the sample solutions for quenching experiments was maintained constant at 1.0 M using sodium chloride (NaCl). The Stern-Volmer constants were deduced by least squares fitting the data using Sigma plot software.

Circular Dichroism spectra of bLPO were recorded on JASCO, j-600 spectropolarimeter in 100 mm, quartz cell at 25°C in the wavelength region of 200-250 nm. Spectra were accumulated on sample concentration of 2 µM in 5mM sodium phosphate buffer pH 7.0. The CD data are reported as Δε M⁻¹cm⁻¹ values. [29].

bLPO contains 15 cysteins [14], twelve of them are in the disulphide bridges, whose locations are the following [23]; C32-C45, C146-C156, C150-C174, C254-C265, C475-C530 and C571-C596. Denaturation measurements were therefore carried out on DTT treated samples [30].

### 3.4 Analysis of the Steady-State Fluorescence Quenching Data:

Fluorescence quenching involves three types of quenching mechanisms: Dynamic quenching due to time dependent diffusive collisions between the fluorophore and the quencher; static quenching as a result of formation of non-fluorescent complex between the fluorophore and the quencher; and combined dynamic and/or static quenching of fluorophores differing in accessibility to the quencher. Stern-Volmer equations for the quenching mechanisms have been discussed in detail [46]. When static quenching contributes to the dynamic quenching process, upward curving Stern-Volmer plots are obtained. Ksv values can be deduced by using modified Stern-Volmer equation: [49]

\[
\frac{F_0}{F} \exp (V[L_0]) = 1 + K_{sv}[L_0]
\]

where \(F_0\) and \(F\) are the fluorescence intensities in absence and presence of the quenching ligand respectively. \(K_{sv}\) is the Stern-Volmer and \([L_0]\) represents the quencher concentration. \(V\) is the parameter that takes care of the static quenching component. For the case of a heterogeneous, independent system of fluorophores as in a many tryptophan protein such as bLPO, a modified form of Stern-Volmer equation also called Lehrer equation is used to analyse the quenching data [38,50]. An expression for the modified Lehrer plot can also be represented as
\[ F_0 - F / F_0 = f_\lambda K_{en} [L_0] / (1 + K_{en} [L_0]) \]  

(2)

3.5 Distance Measurements:

In Forster’s theory of resonance energy transfer [42], the efficiency of energy transfer (E) from the donor to the acceptor groups is

\[ E = 1 - \phi_P / \phi_a \]  

(3)

where \( \phi_P \) and \( \phi_a \) are the absolute quantum yields of the donor in presence and absence of the acceptor respectively. The efficiency \( E \) is related to the distance \( r \) between the donor (Trp) and acceptor (heme) groups as

\[ r = R_0 (1 - E / E)^{1/6} \]  

(4)

where \( R_0 \) is the Foster distance at which the donor fluorescence is quenched by 50%. \( R_0 \) is defined by the equation:

\[ R_0 = 9.79 \times 10^3 (\kappa^2 \phi_a J n^{-4})^{1/6} \]  

(5)

where \( \kappa^2 \) is the relative orientation between the donor and acceptor transition dipole moments and it varies from 0 to 4 [41]. \( n \) is the refractive index of the medium, taken to be 1.4 [42] and the spectral overlap integral, \( J \), between the tryptophan emission and heme absorption is described by the equation:

\[ J = \int F_\lambda \varepsilon_\lambda \lambda^4 d\lambda / \int F_\lambda d\lambda \]  

(6)

where \( F_\lambda \) is the donor emission intensity at wavelength \( \lambda \) (nm) and \( \varepsilon \) is the acceptor extinction coefficient at \( \lambda \) (nm). The value of \( J \) was determined numerically over the spectral region from 300 to 550 nm.

3.6 Analysis of The Transition Curves:

The free energy of unfolding at each concentration of denaturant was calculated by using the following equation:
\[ \Delta G = -RT \ln \left( \frac{(X_n - X_i)}{(X_i - X_d)} \right) \]  

\( X_i \) is the numerical value of the structure sensitive parameter, \( \Delta \varepsilon \) M\(^{-1}\) cm\(^{-1}\) at 22 nm and \( F/F_0 \) respectively at the ith denaturant concentration; \( X_n \) and \( X_d \) are the numerical values of the same parameter relative to native (n) and the denatured (d) states, respectively. Assuming that the unfolding reaction follows a two-state model

\[ N \leftrightarrow U \]  

where \( N \) is the native protein, \( U \) is the unfolded protein and \( K = [U]/[N] \). Given that

\[ K = \exp \left( -\frac{\Delta G}{RT} \right) \]  

and assuming that

\[ \Delta G = \Delta G^{\text{H2O}} - m[\text{denaturant}] \]  

The \( \Delta G^{\text{H2O}} \) was estimated by least squares fit of \( \Delta G \) vs [denaturant] data [29].

3.7 Results and Discussion:

Figure 3.1 shows the corrected steady state emission between 300 nm and 550 nm of native bLPO, following excitation at 280, 290 and 295 nm. Emission maximum is observed at 338 nm with a band width of 52 nm. This agrees with the fluorescence spectrum of bLPO first reported by Makinen and Makinen [31]. Low intensity observed on excitation at 295 nm shows that tyrosine residues on excitation of the sample at 280 and 290 nm transfer their energy to tryptophan residues, but they are not excited at 295 nm [41]. This was apparent when the sample was excited at 300 nm (data not shown) and gave identical emission profile as at 295 nm. In addition, the excitation spectrum (not shown) has a peak at 280 nm with shoulders at 290 and 294 nm. These features suggested that that the observed fluorescence at \( \lambda_{\text{em}} = 338 \) nm, arises nearly entirely from the from the tryptophan residues and that the contribution from the tyrosine residues, if any, must be negligibly small. The emission maximum for bLPO is considerably red shifted, compared to those in CCP, HRP, and Cyt-
Figure 3.1: Intrinsic fluorescence of bovine lactoperoxidase in 5 mM sodium phosphate buffer pH 7.0; The data points are the fluorescence intensity corrected for inner-filter effects and normalized for the absorption at excitation wavelength. The stars represent emission spectrum at $\lambda_{ex} = 280$ nm; The filled diamonds represent the emission spectrum at $\lambda_{ex} = 290$ nm and solid line represents the emission spectrum at $\lambda_{ex} = 295$ nm.
oxidase, observed at 324 and 328 nm respectively (table 3.1). The average environment around tryptophans in later proteins is suggested to be predominantly hydrophobic. This suggests that in bLPO, the tryptophans are approximately symmetrically distributed in hydrophobic as well as in hydrophilic environment, hydrogen bonded to polar acid residues [41] and/or solvent water. Figure 3.2 shows a schematic representation of the bLPO heme and tryptophan residues in their relative orientation as deduced from the theoretical three dimensional model for bLPO [23]. The model shows that the single polypeptide is nearly spherical in shape of approximately of 64 Å diameter. The heme is deeply buried inside the peptide fold and is located approximately at the centre. Figure 3.2 shows that many of the tryptophans, except W610 at 9.97 Å from the ferric ion, are located close to or on the protein surface.

Table 3.1 shows the fluorescence data of bLPO derivatives. The emission maximum of cyanide derivative of bLPO also does not experience any shift. Native bLPO has ferric ion in high-spin state which is changed to low spin state on cyanide ion binding in the sixth co-ordination position [16,20]. Emission maxima of the compound II of LPO obtained by reacting it with one equivalent of H₂O₂ and its sulf derivative, called self heme-containing LPO [51] show ~5 nm red shift. In LPO compound II, the iron is in formal IV valence state, which is reduced back to ferric state on reaction with hydrogen sulphide to obtain sulf derivative [51]. The small red shift may not be due to spin-state of heme iron but may be due to small conformation change in the vicinity of the heme group brought about by formation of iron (IV) as well as its sulf derivative. The overall observation, however, is that heme has very little contribution in fluorescence emission in bLPO.

The Forster’s theory of resonance transfer defines the parameters that relate the efficiency of energy transfer to the distance and orientation between the tryptophan donor and heme acceptor groups (see Materials and Methods). To determine distance between a donor-acceptor pair, their relative orientation must be known. The value for the orientation parameter κ² lies between 0 to 4 [41]. Its value can be estimated from the x-ray structure data of the protein. However, it can not be estimated for bLPO, tryptophan moiety possess two perpendicular emission dipoles. The heme group also contains electronic transitions along two perpendicular axes. Tryptophans in
Figure 3.2: Computer graphics display of the twelve tryptophans showing their relative orientations and distances from the centrally located ferric protopheme IX. This molecular graphics image was produced using the Insight-II graphical display software from the Molecular Simulations Incorporated, M.C.I., San Diego, U.S.A.
hydrophobic environment are expected to experience restricted rotation. Similarly the heme group in bLPO is also expected to show considerable rotational mobility as it is deeply buried at the hydrophobic centre [23]; although some restricted rotation is to be expected, as it is linked to the peptide through two ester bonds [19].

<p>| Table 3.1: Fluorescence Data of LPO Derivatives and other Heme Proteins |
|---------------------------|----------------|----------------|----------------|----------------|----------------------------------|</p>
<table>
<thead>
<tr>
<th>Protein</th>
<th>( \lambda_{em} )</th>
<th>Soret ( \lambda_{max} )</th>
<th>( \phi(\text{AcTrpNH}_2) )</th>
<th>( \phi(\text{absolute}) )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine LPO</td>
<td>338</td>
<td>412</td>
<td>0.142</td>
<td>0.0185</td>
<td>this work</td>
</tr>
<tr>
<td>LPO-CN</td>
<td>338</td>
<td>426</td>
<td>0.148</td>
<td>0.0193</td>
<td>this work</td>
</tr>
<tr>
<td>LPO II/S</td>
<td>343</td>
<td>412</td>
<td>0.0209</td>
<td>0.00273</td>
<td>this work</td>
</tr>
<tr>
<td>HRP</td>
<td>328</td>
<td>403</td>
<td>0.039</td>
<td>0.005</td>
<td>[39]</td>
</tr>
<tr>
<td>CCP</td>
<td>324</td>
<td>408</td>
<td>0.07</td>
<td>0.009</td>
<td>[35]</td>
</tr>
<tr>
<td>Cyt-oxidase</td>
<td>328</td>
<td>422</td>
<td>0.5</td>
<td>0.065</td>
<td>[37]</td>
</tr>
</tbody>
</table>

It was therefore, in the present study, that the average of \( \kappa^2 \), taken over all orientations of the 15 tryptophan-heme pairs in the molecule, to be \( 2/3 \). Also, since the sixth root is involved in the calculation of \( R_0 \), variation of \( \kappa^2 \) from 1 to 4 results in only a 26% error in \( r \) and for fractional value, the error involved may be still lower; and further, for random orientation of donor and acceptor, \( \kappa^2 = 2/3 \) is generally assumed for proteins [43]. The refractive index, is generally given a value of 1.4 for proteins [42] and \( \phi_a = 0.2 \), the absolute quantum yield for tryptophans in absence of acceptor, estimated for apo-hemoglobin [44] was assumed [35,37]. The value \( J \) for tryptophan emission and heme absorption in bLPO was numerically estimated to be \( 8.63 \times 10^{-14} \text{cm}^3 \text{M}^{-1} \). The \( R_0 \) value for bLPO was calculated to be \( 36.8 \text{ Å} \). The value of \( r \) was deduced to be equal to \( 25.1 \pm 0.2 \text{ Å} \) using equations 3 and 4 and using \( \phi_p = 0.0185 \pm 0.0005 \) (table 3.1). The value of \( r \) here represents “average” distance between the heme and tryptophan residues with bLPO and gives qualitative estimate of the distance over which energy transfer occurs.
Using the theoretical three dimensional model for bLPO [23], it is possible to estimate relative contribution of each tryptophan-heme pair to the observed fluorescence intensity, as has recently been estimated for CCP [35]. Combining equations 3 and 4 (Materials and Methods), \( R_0 = 36.8 \text{ Å} \) and using the distance of each tryptophan from the heme center from figure 3.2, the relative quantum yield, \( \Phi_{\text{p}}/\Phi_{\text{a}} \) for individual tryptophan were estimated. The percent contribution of each tryptophan to the total fluorescence of bLPO is defined by

\[
\%\Phi = \frac{(100 \times (\Phi_{\text{p}}/\Phi_{\text{a}}))}{\Sigma(\Phi_{\text{p}}/\Phi_{\text{a}})}
\]

(10)

Calculated value of \( 1/12 \Sigma(\Phi_{\text{p}}/\Phi_{\text{a}}) = 0.11 \) agrees well with the observed value of \( \Phi_{\text{p}}/\Phi_{\text{a}} = 0.0925 \), deduced from the fluorescence intensity of bLPO. In the three dimensional theoretical model of bLPO, Trp2, Trp91, and Trp 119 were omitted [23]; the agreement therefore, suggests that the contribution of the three tryptophans, omitted in the model may be small. The \( \%\Phi \) values in the table 3.2 clearly show that Trp337 located 31.18 Å and Trp404 located at 39.59 Å from the ferric centre apparently together dominate the steady-state fluorescence of bLPO, contributing 66% of the observed intensity. Table 3.2 also shows that the fluorescence intensity of all other tryptophans in the protein is quenched differently depending on their proximity to the heme group and their relative orientation.

3.8 Denaturant-induced conformational changes in bLPO:

The change in intensity of fluorescence emission of DTT treated bLPO was measured in the presence of increasing concentrations of denaturing agent GdnHCl, which provides information on the stability of the protein structure [40] and the environment of the fluorophores [41]. In absence of GdnHCl, the emission maximum located at 338 nm for bLPO (table 3.1) was found to be red shifted to 356 nm. This indicates that the tryptophans, buried in the hydrophobic environment in the native folded state and inaccessible to solvent water get exposed to excess environment. In figure 3.3 is plotted the fluorescence intensity relative to that in the absence of GdnHCl, \( (F/F_0) \), against [GdnHCl]. It shows a typical sigmoidal nature of the
Figure 3.3: Effect of GdnHCl concentration on the fluorescence intensity of bLPO, measured at $\lambda_{ex} = 295$, and emission maximum $=338$ nm. Fluorescence intensity is normalised to the fluorescence intensity of native bLPO in absence of the denaturant ($F/F_0$). The inset shows the plot of $\Delta G$, calculated from equation 7, vs denaturant concentration. The smooth curve in the inset was calculated using equation 9.
unfolding process for bLPO, characteristic of a simple two-state transition between the native (N) and unfolded (U) forms of the protein. The inset in figure 3.3 shows that the free energy change varies linearly with the denaturant concentration.

Denaturation of DTT treated bLPO was also followed by CD spectra in backbone region. Figure 3.4 shows the CD spectra of bLPO in absence and presence of 9.5 M urea. The CD spectrum in absence of urea for DTT treated bLPO and native bLPO were almost overlapping (data not shown). Thus DTT treatment breaks disulphide linkages but also helps to maintain, overall conformational integrity in bLPO. This CD spectrum (Figure 3.4) agrees with the CD data in the backbone region of bLPO, reported earlier [13]. The data is also very similar to the CD of HRP-C in the backbone region [36]. Sievers [13] showed that the two maxima around 206-208 nm and 220-224 nm are due to 23% helix, 65% β sheet and about 12% random coil secondary structure (Figure 3.4). However, even at 9.5 M urea, some residual secondary structure remains as suggested in figure 3.5, where the observed Δε at 222 nm data are plotted against urea concentration. The Δε does not seem to have approached a constant value up to 9.5 M urea. HRP-C also showed similar behaviour [45,36]. Like fluorescence quenching experiment, sigmoidal profile observed in figure 3.5 is consistent with a simple two state transition between native and denatured form of the protein. The denaturing curves were analysed in terms of the free energy of unfolding (ΔG) which is assumed to be linearly dependant on the denaturant concentration [29]. The ΔG^H2O obtained from CD measurements is of longer magnitude than obtained from the analysis of fluorescence emission data (Table 3.3). Neutral urea is known to be 1.5 to 2.5 times less effective as denaturant than GdnHCl [29]. Probably therefore, the unfolding by urea in CD experiment is somewhat incomplete and may be partly responsible for the difference in the computed ΔG^H2O values in the two experiments. The values in the table 3.3 show that the mid point concentration of ~5.5 M in both the experiments, suggested that the mechanism of binding by urea and guanidine hydrochloride to BLPO and their mechanisms of denaturation of bLPO may be very similar. The m value is the measure of the cooperatively of the denaturation. These observations suggest that the conformational states probed by tryptophan fluorescence and those probed by the circular dichroism of
Figure 3.4: Dependence of the backbone CD of bLPO on urea concentration during denaturation. The structure sensitive CD parameter, $\Delta \varepsilon$ M$^{-1}$ cm$^{-1}$ at 222 nm plotted against urea concentration. The smooth curve was obtained using equation 7, and using parameters fitted to equation 9.
backbone amide chromophores are equally accessible and show almost same cooperativity.

| Table 3.2: Percent Quantum Yield for Tryptophan Residues in Native bLPO (%\(\Phi\)) |
|---------------------------------------------|----------------|----------------|
| Trp\(^a\) | r A\(^d\) | %\(\Phi\) |
| Trp163 | 18.77 | 1.31 |
| Trp178 | 23.2 | 4.47 |
| Trp220 | 14.93 | 0.34 |
| Trp337 | 31.18 | 20.41 |
| Trp404 | 39.59 | 45.84 |
| Trp441 | 21.81 | 3.14 |
| Trp485 | 26.15 | 8.63 |
| Trp501 | 22.39 | 3.65 |
| Trp569 | 18.26 | 1.11 |
| Trp610 | 9.97 | 0.03 |
| Trp646 | 20.93 | 2.48 |
| Trp647 | 26.12 | 8.58 |
| SUM | | 100 |
| OBS | | 100 |

| Table 3.3: Thermodynamic data on the denaturation of bLPO |
|---------------------------------------------|----------------|----------------|
| Probe | \(\Delta G^{H2O}\) kj / M | m (kM \([\text{Mprot}][\text{Mden}]^{-1}\)) | \(r^2\) | [denaturant]_{1/2} M |
| Tryptophan fluorescence | 8.7 | 2.1 | 0.93 | 5.5 |
| Backbone CD | 13.9 | 2.2 | 0.95 | 5.6 |
3.9 Enzyme Fluorescence Quenching by Neutral and Ionic Quenchers:

Polar, uncharged compound, acrylamide, and ionic quenchers, I⁻ and Cs⁺ were each examined for their ability to quench the intense fluorescence of bLPO. Figure 3.6 shows the fluorescence quenching by acrylamide. The upward curving plot for acrylamide indicates that all the tryptophan residues are nearly accessible and a dynamic quenching, having static component to the quenching process [47]. The static quenching indicates that there is an association between the quencher and the fluorophore such that collisional process is instantaneous. In such cases the Stern-Volmer constant (Ksv) which is a measure of dynamic quenching, was derived from the modified Stern-Volmer plot [47], (eqn.1). The value of Ksv = 8.4 ± 1.4 M⁻¹ was deduced for acrylamide quenching process. The Ksv value in multi-tryptopham proteins such as bLPO essentially denoted the weighted average of the individual quenching constants.

Like acrylamide, iodide ions also quenched the bLPO fluorescence. Steady-state quenching of bLPO fluorescence by iodide ion however, did not give satisfactory linear plot of F₀/F vs [L₀]. In figure 3.7, the fluorescence intensity expressed as F₀-F/F₀, and plotted as a function of the quencher concentration, is seen almost to follow equation 2. This indicates, the quenching process to be dynamic due to free diffusion of iodide ion. Iodide and thiocyanate ions are the functional substrates and have been shown to bind native bLPO forming 1:1 complex [6,7,21]. Absorption spectrum of bLPO in presence of various amounts of iodide and thiocyanate ions, up to 0.7 M (data not shown) showed, that the same maxima in both uv and visible region, including Soret absorption maximum are present. The intensities of Soret and other bands in the visible region are, however, lowered, but not shifted up to 0.7 M (see figure 3.7) of the anion concentration (see also [16,21] and references there in). The optical spectrum of bLPO being due to π-π* transitions in the porphyrin ring, and to the charge transfer between macrocycle itself and Fe (III) ion, the interaction of anions, like iodide and thiocyanate ions, is strongly correlated with the nature and the strength of the interaction in the vicinity of the heme moiety and not with the conformational changes elsewhere in the protein [16,21]. The fluorescence emission maxima in table 3.1 show that heme itself has only one small contribution, if any, in the fluorescence. Also, the
Figure 3.5: Effect of urea denaturation on the CD spectrum of DDT treated bLPO. Urea indicates samples treated with 9.5 M urea in 9.5 mM sodium phosphate buffer pH 7.0. Native bovine LPO is designated as bLPO in 5 mM sodium phosphate buffer pH adjusted to 7.0.
Figure 3.6: Steady-state fluorescence quenching of bLPO by acrylamide. The fluorescence was measured in 5 mM phosphate buffer pH 7.0 as described in Materials and Methods. The fluorescence intensity is expressed as the fraction of unquenched fluorescence, $F_0$ at 295 nm excitation and 338 nm emission wavelength.

Solid circles are data points and the smooth curve was obtained using equation 1, and using fitted parameters.
absorbance of bLPO at the excitation wavelength was maintained \( \leq 0.03 \), thus the absorbance changes in presence of the anions were not measurable. The quenching effects however, are effectively being measured for anion bound bLPO form. The net positive charge on the surface of the bLPO molecule may have facilitated the diffusion. The figure 3.7 also indicates that the fluorescence of the bLPO in presence of differing amounts of iodide ion, is essentially heterogeneous with tryptophan residues widely differing in accessibility [38,50,47]. The data were analysed using the modified Lehrer equation, (equation 2). A unique set of parameters \( (K_v, f_a) \) were obtained with the value of \( K_v = 4.05 \pm 0.64 \) and the value of the fraction of the protein fluorescence accessible to the quencher, \( f_a = 0.52 \pm 0.03 \). The solid line shows the curve calculated using fitted parameters.

Thiocyanate ion is very similar to iodide ion in binding to bLPO [7,21]. However, relative fluorescence intensity did not show any systematic variation with the quencher concentration. The ineffectiveness of thiocyanate ion as a quencher, may be therefore, due to its linear shape, not conducive enough for effective collisional quenching as is the iodide ion [41].

Cs\(^+\) also did not quench the fluorescence, up to a concentration of 0.7 M. The effect of Cs\(^+\) was only to produce downward curving Stern-Volmer plot with considerable scattering in the data, indicating that for bLPO, Cs\(^+\) is a very inefficient quencher [46]. LPO with pi > 8 [14] carries net positive charge on the surface. This may have inhibited the diffusion of Cs\(^+\) and ions and hence the quenching of tryptophan fluorescence.

The \( K_v \) values for fluorescence quenching by acrylamide and iodide suggest that the surface of bLPO molecule is sufficiently conducive for interactions with substrates with hydrophobic and hydrophilic character. Interactions with ionic substrates such as iodide and thiocyanate ions are functional interactions. Presence of substantial hydrophobic areas on the surface of bLPO are probably due to its mammalian origin, although, unlike mammalian membrane proteins such as cytochrome oxidase [37], it is active in aqueous solutions. The aqueous solutions however, are relatively unstable and become turbid due to aggregation. Hydrophobic materials such as teflon or glass in contact with the enzyme increased the tendency of
Figure 3.7: Modified Lehrer plot of the steady-state quenching of bLPO by iodide ion. The decrease of fluorescence \((F_0 - F)\) at 295 nm excitation and 338 nm emission wavelength is expressed as ratio of \((F_0 - F)\) to the unquenched fluorescence \(F_0\) and plotted as a function of iodide ion concentration. The smooth curve calculated using parameters fitted to equation 2.
aggregation; besides bLPO also shows a high tendency to strongly adhere to surfaces such as glass [26]. These properties are characteristic of bLPO, parallel our observations to show that the surface of bLPO molecule is equipped for both ionic and hydrophobic interactions.

3.10 CONCLUSIONS

The observed characteristics of the intrinsic fluorescence of bLPO allowed us to assign the emission signal entirely to the tryptophan residues. The fluorescence maximum observed at 338 nm revealed that the 15 tryptophans in the bLPO molecule, are equally distributed in both hydrophobic and hydrophobic environments. The fluorescence is quenched due to energy transfer from tryptophans to heme chromophore. The “average” distance between heme and the tryptophan within the enzyme is $25.1 \pm 0.2 \AA$. Relative quantum yield, $(\Phi_r/\Phi_a)$ for each tryptophan was computed from the theoretical three-dimensional model for bLPO. The total relative quantum yield averaged over the twelve tryptophans, (0.11) agrees well with the measured quantity in the present work (0.0925). Effects of GdnHCl on fluorescence is to expose the tryptophans to aqueous solvent. Due to the presence of twelve disulphide residues in bLPO, fluorescence and backbone circular dichroism spectra of DTT treated bLPO in presence of urea and GdnHCl showed that bLPO is as conformationally stable as plant peroxidase HRP-C. Tryptophans are well distributed in both hydrophobic and hydrophilic regions of the molecule and along with amide chromophores show equal accessibility and co-operativity in the interaction of the denaturants with bLPO. Both ionic iodide ion and neutral acrylamide quenched the fluorescence of LPO, however, Cs$^+$ did not quench. The quenching data analysed by modified Stern-Volmer and Lehrer equations, revealed that Stern-Volmer constants are of comparable magnitude indicating that bovine LPO is equipped for both hydrophobic and hydrophilic interactions.
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