CHAPTER-IV

STUDIES OF THE INTERACTION OF ORAL IRON CHELATOR DEFERIPRONE (L1) WITH HEMOGLOBIN, THE MAJOR RED BLOOD CELL PROTEIN
INTRODUCTION:

Iron chelators are widely used in attempts to prevent long-term consequences of iron overload in some patients like thalassemia and sickle cell anemia. The excess iron acquired through long term blood transfusions and results in damage to liver, endocrine organs and heart. Several studies have highlighted the importance of sustained reduction of body iron burden as the principal determinant of clinical outcome in these disorders. Deferoxamine (DFO) is the most widely used iron chelating agent and is being used during last 30 years. But it has significant disadvantages, including its high cost, parenteral method of administration, toxicity and frequent non-compliance, leading to a search for safe, orally active alternative iron chelator deferiprone (L1) [178]. The oral iron chelator [179], deferiprone (1,2-dimethyl-3-hydroxy-pyrid-4-one) is currently the most effective drug for the treatment of transfusional iron overload in β-thalassemic patient [180]. Iron chelators have been shown to inhibit the production of oxygen radical species resulting in decreased lipid peroxidation and oxidative DNA damage both in vitro and in vivo [181-2]. Both iron chelator deferoxamine (DFO) and deferiprone (L1) have high iron affinity and specificity, but in comparison to deferiprone, DFO has poor cellular permeability. When compared to deferoxamine, deferiprone has potentially nine fold greater capacity to permeate cell membranes [183].

Different toxic effects are being noticed worldwide in case of long term use of the iron chelator deferiprone (L1). The major toxicity reported presently due to the use of iron chelator deferiprone (L1) includes severe neutropenia, agranulocytosis, arthropathy, gastrointestinal intolerance and liver enzyme abnormalities [184,185]. Recently it has been reported that deferiprone (L1) potentiates oxidative DNA damage in iron-loaded liver cells [186]. This iron chelator is presently being studied in clinical trial and appears to be effective with an acceptable toxicity profile.

No much work has been done on the interaction of deferiprone with biological macromolecules. Since deferiprone penetrates through cell membrane it has every possibility of interacting with this protein within RBC and influencing its function. Attempt has been made here to study the effect of deferiprone on hemoglobin, the major functional protein of red blood cell. The conformation of a protein is guided solely by its amino acid sequence and the folding of the polypeptide chain to form native three dimensional structure of protein is a spontaneous process but there are
small molecules, called ligands, which can interact with proteins, modify their conformations, and influence their biological functions.

This chapter of the thesis is designed to investigate whether the drug deferiprone has any interaction with hemoglobin, or whether it can modify the activities of this oxygen carrying molecule, since tetrameric hemoglobin, the allosteric protein binds four oxygen molecules and has an important role in transporting this oxygen from lung to different tissues. To understand this ligand receptor interaction biophysical techniques like fluorescence spectroscopy and circular dichroism has been used.

Fluorescence spectroscopy is now a days used as a versatile tool for the study of biological systems. The absorption of a photon is followed by the emission of light at longer wavelength in some particular molecules, that emission is called fluorescence. Fluorescence of proteins are mainly observed due to tryptophans acting as intrinsic fluorophors when they are contained in the protein molecule and have maximum quantum yield compared to other aromatic amino acids (tyrosine and phenylalanine).

The environment that surrounds an excited chromophore can influence its fluorescence in several ways. Reorientation of the charged group partly determines the amount of energy dissipated in the radiative transition from excited state to the ground state. Charged groups provide interactions that enhances the rate of internal conversion and influence the amount of fluorescence emitted. Both of these processes rely on the fact that the excited state lifetime is long compared to the period of molecular vibration, the fluorescence spectrum is more sensitive to its surrounding than its absorption spectrum.

Indole ring of tryptophans undergo \( \pi \rightarrow \pi^* \) transition upon absorption of a photon. Reorientation of the charged group in the vicinity of the excited molecule results in a decrease in energy and a shift of the emitted fluorescence transition to longer wavelength. Tryptophan of protein molecule can therefore be taken as a very nice probe to detect the conformational change of the molecule around the tryptophans.

The measurement of circular dichroism spectra as a tool to investigate the structural organization of protein molecules is of particular advantage for hemoglobin. These molecules contain, in addition to the protein moiety, the heme, with electronic transitions that are quite intense, diverse and very sensitive both to the surrounding environment and to the ligand binding. This situation offers three distinct regions of
investigation, each containing information concerning a part of the structural organization of the hemoglobin molecule.

In the far UV region from 190 nm to 240 nm the predominant chromophores are the peptide groups correlated with the general tridimensional organization of the molecule.

The near UV region is from 240 nm to 300 nm. In this region the predominant chromophores are the aromatic amino acid side chains, which may give detailed information on "local" chain-chain interactions.

No amino acid chromophore contributes directly to the region above 300 nm including visible but the CD spectra of heme transitions that occur here are governed by asymmetry of protein environment.

The CD spectra between 200 nm to 245 nm are usually reported as mean residue ellipticities in which the concentration is the mean residue molecular weight (MRW), i.e. the molecular weight of the protein divided by the number of amino acid residues per molecule. This is a convenient choice over the molar ellipticity because in this region the contributing chromophores are peptide bonds. The CD spectra of peptides are in the three fundamental forms, α-helix, β-structure and random coil. The first and simplest method derived the fractional value of helical residues from CD through at 222 nm [94].
RESULTS:

Figure 4.1 shows the quenching of tryptophan fluorescence of hemoglobin at different concentrations of deferiprone. Fluorescence intensities of tryptophans of hemoglobin were quenched significantly, revealing their interaction with deferiprone. The inset of figure 4.1 shows quenching of the fluorescence of aqueous solutions of free tryptophans by deferiprone (L1). The tryptophans of the protein hemoglobin excited at 280 nm have a shorter $\lambda_{\text{em}}$ (331 nm) than does free tryptophan (351.8 nm) in polar aqueous medium excited at the same wavelength. This shift is a result of shielding of the tryptophan residue from water by a protein matrix. The tryptophans in hemoglobin are in less polar or non-polar environment compared with free tryptophans in aqueous solvent [187]. With gradual addition of deferiprone (L1) the fluorescence intensity of the treated hemoglobin decreases [Fig 4.2]. It is interesting to mention here that the tetrameric hemoglobin molecule contains six tryptophan residues. Fluorescence measurements reveal that there is a significant interaction of deferiprone with tryptophan residues of hemoglobin. Thus present data may be indicative of the feature that tryptophan is at or near the possible binding site.

Figure 4.3 represents the fluorescence quenching of globin solution (conc. $1.2 \times 10^{-6}$ M) when various amount of deferiprone (L1) were added to it. It is interesting to note that fluorescence of globin is quenched in the same manner as in case of hemoglobin. This process of quenching of tryptophan residues of hemoglobin by deferiprone (L1) occurs due to the interaction of the drug molecule with the globin moiety of hemoglobin.

Quenching of fluorescence intensity of a fluorophore occurs as a consequence of two processes: (1) Bimolecular collisional encounter (called dynamic quenching) between the fluorophore and the quencher molecule leading to energy transfer. (2) Ground state complex formation between the fluorophore and the quencher (called static quenching) leading to the reduction in effective number of excitable fluorophors. In the static quenching process, the fluorophor in its ground state forms complex with quencher molecule resulting in the reduction of effective number of excitable fluorophors. Only the free uncomplexed fluorophores are excitable by absorbing the light intensity and take part in the further deactivation process. Quantum yield in presence and in absence of the quencher remains same in case of static quenching and
Fig 4.1: Quenching of tryptophan fluorescence of hemoglobin (8 μM) by deferiprone. Fluorescence intensity of untreated hemoglobin is taken as 100%. Inset shows the quenching of aqueous solution of free tryptophans of concentration corresponding to that in hemoglobin by deferiprone.
Fig 4.2: Typical fluorescence emission spectra of hemoglobin in presence of different deferiprone (L1) concentration.

(a) no deferiprone (L1) [1]

(b) with addition of increasing concentration of deferiprone (L1) in μM [2-15]
Fig 4.3: Typical fluorescence spectra of pure globin in presence of different deferiprone (L1) concentration
(a) no deferiprone (L1) [1]
(b) with addition of increasing concentration of deferiprone (L1) in μM [2-9]
hence lifetime of the excited state remains same in presence and in absence of quencher.

Figure 4.4 is the simple Stern-Volmer plot of \( F_0/F \) versus deferiprone concentration (L1) where \( F_0 \) and F are the fluorescence intensities of tryptophans of hemoglobin in absence and in presence of deferiprone (L1). \( F_0 \) is taken to be 100% always. This plot has been found to be linear.

Figure 4.5 is the linear plot of \( 1/\Delta F \) versus \( 1/L_4 \) following the equation [188],

\[
1/\Delta F = 1/\Delta F_{\text{max}} + 1/KL_4 /1/\Delta F_{\text{max}}
\]

Where \( \Delta F = F_0 - F; \) \( F_0 \) and F represents the fluorescence intensity of hemoglobin in absence and in presence of deferiprone. \( \Delta F_{\text{max}} \) is the maximum change in fluorescence intensity. The intercept of the above plot on the \( 1/\Delta F \) axis corresponding to \( 1/ L_4 = 0 \) measures \( 1/\Delta F_{\text{max}} \) and the slope gives the estimate for the affinity constant \( K \).

**Table 4.1: Dependence of the binding constant (K) on the concentration of NaCl solution.**

<table>
<thead>
<tr>
<th>NaCl (M)</th>
<th>Affinity constant K ((\times10^3) \text{ M}^{-1}) for deferiprone binding to hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.075</td>
<td>8.64</td>
</tr>
<tr>
<td>0.15</td>
<td>4.54</td>
</tr>
<tr>
<td>0.25</td>
<td>1.87</td>
</tr>
<tr>
<td>0.30</td>
<td>0.40</td>
</tr>
</tbody>
</table>

The binding constant for deferiprone to hemoglobin in 0.15M NaCl was found to be \( 4.5\times10^3 \text{ M}^{-1} \) (table 4.1). Another significant feature of this interaction is that the binding affinity constant \( K \) changed significantly with increasing NaCl molarity in the range 0.075 M to 0.30 M. This can be attributed to the fact that electrostatic interaction might play a significant role in the formation of the binding complex. The competition of sodium ions would then be responsible for the decrease in binding constant with an increase in salt concentration.
Fig 4.4: Stern-Volmer plot of $F_0/F$ vs. deferiprone concentration

$F_0$ stands for the fluorescence without any deferiprone and $F$ stands for the fluorescence value after addition of deferiprone at each data point.

Concentration of deferiprone (L1) [micromolar]

$F_0$ stands for the fluorescence without any deferiprone and $F$ stands for the fluorescence value after addition of deferiprone at each data point.
Fig 4.5: Plot of $1/\Delta F$ vs. $1/L_t$, where $L_t$ is the concentration of deferiprone added to the tetrameric hemoglobin.
Table 4.2: Percent of α-helix of deferiprone (L1) treated hemoglobin at different Drug/Protein ratio.

<table>
<thead>
<tr>
<th>Drug/Protein (D/P)</th>
<th>Percent of α-helix remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>75.00</td>
</tr>
<tr>
<td>4</td>
<td>71.80</td>
</tr>
<tr>
<td>8</td>
<td>62.98</td>
</tr>
<tr>
<td>12</td>
<td>59.10</td>
</tr>
</tbody>
</table>

Figure 4.6 shows percentage helicity of hemoglobin in presence of increasing concentration of deferiprone. In the wavelength region 205 nm - 250 nm CD spectrum of a protein gives information about its conformation in relation to the secondary structure. Untreated hemoglobin shows its characteristic CD spectrum asserting its 75% α helix content with negative ellipticities at 208nm and 222nm. These two ellipticities gradually decreased with increasing deferiprone concentration. Thus the (α-helix content of the drug-treated hemoglobin gradually decreases relative to the untreated protein indicating a conformation change of the macromolecule induced due to its interaction with deferiprone.

In order to investigate whether deferiprone binding to hemoglobin has any effect on the oxygen binding capacity of hemoglobin, the release of oxygen was studied at varying [Drug]/[Protein] ratio, denoted by D/P ratio; by Gilson 5/6 Oxygraph instrument. Experiment was designed with the hemoglobin in 100%-oxygenated form. 2ml of Hb was taken in the cell of the instrument provided with Clarke electrode. deferiprone was added in the cell with constant stirring so that D/P ratio was varied from 0-12. It was observed that in presence of the drug no oxygen was released from hemoglobin. This result is indicative of the fact that deferiprone binding to hemoglobin does not affect the oxygen binding motif of this allosteric molecule.
Fig 4.6: Plot of percent $\alpha$ helicity of tetrameric hemoglobin as a function of deferiprone concentration.
DISCUSSIONS:

Oral iron chelator deferiprone effectively removes abnormal free iron deposits from membranes of intact thalassemic and sickle RBC. But sporadic occurrence of agranulocytosis in association with deferiprone and the highly variable frequency of other possible side effects such as arthralgia have created uncertainty about the true incidence of deferiprone related complications [189]. Therapeutic dose of deferiprone used for iron chelation of thalassemic patients is 75-100 mg/kg body weight/day [190] which corresponds to 600-800 μM concentration of the drug in the body fluid and for all the experiments this drug concentration up to 250 μM has been used which is well within the limit of the therapeutic relevance.

It is well known that hemoglobin binds drugs and other xenobiotics without forming covalent bonds. Now deferiprone has pKₐ value 9.7 [191]. At pH 6.8 the drug will be in the hydroxylated form predominantly. The –OH group of the drug possibly interacts with the amino acid residues neighboring tryptophans in hemoglobin in hydrogen-bonded mode with decreasing interaction affinity when NaCl concentration is increased gradually in the range 0.075M-0.30M. Hemoglobin complexes with bezafibrate and its oxygen affinity are lowered [192]. Another tranquilizing drug chlorpromazine binds with hemoglobin in a positively cooperative manner and releases oxygen from it [193]. Experimental results here suggest that deferiprone also binds with hemoglobin with a binding affinity constant K = 4.5×10³ M⁻¹, mode of binding being hydrogen bond like electrostatic in nature, but the interaction does not release any oxygen from hemoglobin molecule. Linearity of the plot in figure 4.4 also manifests the fact that the interaction occurs with a single mode of binding. Binding of deferiprone modifies the conformation of hemoglobin, percentage of α-helix being reduced with increasing concentration of the drug. Fluorescence of hemoglobin tetramer is mainly due to the six tryptophan residues, since tryptophan has got the highest quantum yield among all the amino acid residues. From the inset of figure 4.1, the estimated value of binding affinity constant for free tryptophan and deferiprone is K =7.69×10³ M⁻¹, which is also of the same order as with the protein itself. Thus, it can be inferred that the aromatic residue tryptophan is at or near the binding site of the ligand.

In vivo, the structure, energetic states and functional properties of hemoglobin molecules are strictly correlated with its solution environment, since the binding is hydrogen bond like electrostatic in nature, interaction between proteins and ligands
demonstrate both steric and physical complementarity between the two. The fall in affinity values with the increase in NaCl upto 0.30M can be attributed to the proportionate decrease in salt link type interactions between the drug and tetrameric hemoglobin due to increased charge shielding by sodium salt over the still remaining tetrameric structure.

Studies on the mode of action of deferiprone induced conformational modification of hemoglobin is of significance from the point of view of toxicity. This study might be of help in further understanding the effect of deferiprone on metabolic processes to assess potential toxicity and safety formulation of the drug.