CHAPTER 5

FLUORESCENCE QUENCHING STUDIES OF BOVINE SERUM ALBUMIN AND INTERACTION WITH HEME AND SYNTHETIC HEME

5.1 Introduction

The term albumin refers to any unconjugated, heat-coagulable protein which is soluble in pure water, as distinct from such water-insoluble proteins as globulin's (salt soluble), glutelins (alkali soluble) and prolamins (soluble in ethanol-water mixtures) [1]. There are various types of albumins existing, such as ovalbumin (from egg white), lactalbumin (from milk and whey), and serum albumin. The best known and the most studied albumin are the Serum or the Plasma albumins, which constitute the major protein component of the blood serum of vertebrates.

The serum albumins belong to a mutigene family of proteins that includes α-fetoprotein (AFP) and mammalian group-specific component (GC) or vitamin D-binding protein [2]. They are relatively large multi-domain proteins, which as the major soluble protein constituents of the circulatory system have many physiological functions. The albumins aid in the transport, distribution and metabolism of many endogenous and exogenous ligands. The serum albumins act as carriers in the bloodstream and heme is also carried to the haemoglobin. When synthetic heme as for example, FeTPPS₄ is introduced at the place of natural heme, the synthetic heme being a foreign body (antigen), the interaction between bovine serum albumin and the synthetic heme is an antibody-antigen interaction [3]. These ligands represent a spectrum of chemically diverse molecules, including fatty acids, amino acids (notably tryptophans and cystein), steroids, metals etc. If one were to obtain blood from the circulation of any of the higher order of animals, and by centrifuging remove from it the formed elements (erythrocytes, leukocytes and platelets), there would remain a
fluid portion called plasma. This clear fluid can be visualised as the extra cellular matrix of the whole blood which may be regarded as a somewhat a typical tissue.

Plasma is an aqueous solution of low and high molecular weight components of diverse composition and function.

The operational difference between plasma and serum is that the latter is devoid of a blood component known as fibrinogen which aids in coagulating the red blood cells. Serum albumins are extremely soluble in water, not precipitated by half-saturated ammonium sulphate but are heat coagulable.

There is a close similarity between the serum albumins of various mammals [1]. All contain a single peptide chain of approximately 500-600 amino acid residues, molecular weight of 65,000 to 70,000, terminates in an aspartic acid residue at the amino end, and in either leucine, alanine or valine at the carboxyl terminus. The bovine serum albumin (BSA), a protein of molecular mass of 66 k Da [15], consists of 585 amino acids and two tryptophans. A total of 61% of the amino acid sequences are conserved among known sequences of bovine [4], rat [5] and human serum albumins [6]. Bovine serum albumin (BSA) has absorbance at $A_{278} = 0.67$, at 1 mg per 1 ml.

Resemblance between human serum albumin and bovine serum albumin is apparent in the amino acid composition as given in table 5.1 [8]. The bovine serum albumin contains a very large number of titrable groups, approximately 100 carboxyl groups [1] (aspartic plus glutamic acid residues, less amide ammonia) and 100 basic groups (arginine, lysine and histidine) plus the phenolic groups of tyrosine. All of the half cystein residues except one that exists in the oxidised state as cystein, thus having 17-18 disulphide cross-linkages into the protein molecule. Only the difference in the reactivity of sulphydryl groups was observed, between the human and the bovine serum albumins, otherwise, the two albumins were having the same components.

The synthetic porphyrin FeTPPS$_4$ which act as model compound for various water soluble heme proteins was prepared [9]. We studied BSA, because it being an excellent heme carrier and studied the type of binding between the protein and the hemin and also the mode of binding with synthetic heme, which can possibly act as a model for artificial blood. The fluorescence quenching technique was used to study the binding mechanism.
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<th>Amino acid</th>
<th>Human</th>
<th>Bovine</th>
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<tr>
<td>Alanine</td>
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<td>Valine</td>
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<tr>
<td>Amide NH₃</td>
<td>34-35</td>
<td>32-33</td>
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Bovine serum albumin was obtained from Armour Pharmaceutical company, Phoenix, Arizona, USA and hemin was a Sigma product. The synthetic porphyrin, iron (III) tetra ammonium tetra (p-sulphophenyl) porphine FeTPPS₄ was prepared in the laboratory [10]. Pyrrole, propionic acid and benzaldehyde were obtained from Quligens, India Ltd and used without further purification. Deionized water was used throughout the experiment.
The parent ligand 5, 10, 15, 20-tetraphenyl porphin was prepared by the method of Adler et al [3]. The metal (Fe) was inserted in the porphyrin, by the general procedure of Adler et al [11].

The metal incorporation was checked spectroscopically and a Soret band at 392 nm and two smaller bands at 528 and 640 nm were obtained, which matched well with the literature [12]. The absorption spectra were recorded on Shimadzu UV-2000 spectrophotometer. Concentration of FeTPPS$_4$ monomer was measured using $\varepsilon_{392} = 15.2 \times 10^4$ M$^{-1}$ cm$^{-1}$ [12] and also for the weaker bands like 528 and 680 nm, the value of $\varepsilon$ are $1.4 \times 10^4$ M$^{-1}$ cm$^{-1}$ and $0.27 \times 10^4$ M$^{-1}$ cm$^{-1}$ respectively [12]. Checked the concentration of protein (BSA) spectrophotometrically taking the absorbance of 1mg/ml solution in 5 mM tris buffer pH 7.76 at $A_{278} = 0.67$ [7]. The hemin chloride was dissolved in 0.01 N alkali, rapidly diluted it ten times with 5 mM tris buffer pH 7.76 and used in this form within about an hour. The concentration was determined spectrophotometrically in 0.01 N sodium hydroxide using a millimolar absorption coefficient of 58.4 mM$^{-1}$ cm$^{-1}$ at 385 nm [13]. The pH of the solutions were maintained slightly alkaline and using 5 mM tris buffer throughout the experiment.

The fluorescence spectra of all the samples were recorded with excitation wavelength at 276 nm, and the emission monochromator scanned the wavelength region between 280 to 450 nm [14].

5.2 Analysis of Fluorescence Quenching Data:

Figure 5.1 shows the fluorescence emission spectrum of bovine serum albumin in 5 mM tris buffer, pH 7.7 and the concentration of the protein was $5 \times 10^{-9}$ M so as to keep the absorbance < 0.03 at 276 nm [16], which keeps the inner filter effect to minimum. Emission occurs at 335 nm on exciting the sample at 280 nm and monochromator scanned the emission in 280 to 550 nm range. Maximum emission intensity was however, observed when sample was excited at 276 nm. All the quenching measurements were therefore, carried out by exciting the sample at 276nm. BSA contains two tryptophans, 20 tyrosines and 27 phenylalanines (Table 5.1). As the protein tyrosine emission occurs at 303 nm, and phenylalanine emission occurs at
Figure 5.1: Fluorescence quenching of bovine serum albumin with synthetic heme, (FeTPPS$_4$) in 5 mM tris buffer pH 7.7; the data points are the fluorescence intensity corrected for inner-filter effects. The excitation and emission slit widths were 0.5 mm. The solid line represents the emission spectrum at $\lambda_{ex} = 276$ nm of native BSA, and the filled diamonds represent the emission spectrum at $\lambda_{ex} = 276$ nm of 0.005 $\mu$M of FeTPPS$_4$ added to native BSA.
282 nm [16], the observed emission at 335 nm is due to the excitation of two tryptophan residues. The tyrosines and phenylalanine on excitation at 276 nm transfer their energy to tryptophan residues. NAT A, (n-acetyl tryptophanamide), a water soluble tryptophan standard gives emission at 356 nm. Considerably blue shifted emission of BSA at 335 nm shows that in BSA, the tryptophans are located in the hydrophobic region not accessible to solvent water easily. Figure 5.1 also shows that the fluorescence intensity is quenched by presence of heme group. In figure 5.2, we have plotted relative fluorescence intensity, $\frac{F_0}{F}$ as a function of the ratio of quencher to protein [Heme]/[BSA]. We see that up a ratio which falls approximately between 1 and 2 heme/BSA, the quenching essentially varies linearly with the ratio and at higher quencher concentration, the curve shows saturation. This can be explained that BSA is predominantly quenched by static quenching process and there are more than one binding sites, but less than two. In other words it can be said, that BSA has one high affinity binding site and also experiences non-specific binding by hemin molecule. The solid line in the figure is only drawn to show the nature of variation of quenching as a function of quencher concentration.

Figure 5.3 shows the quenching of fluorescence of the protein by the synthetic heme. It is seen that the relative fluorescence intensity $(\frac{F_0}{F})$ remains linear up to heme to protein ratio of 1:2, and at higher quencher concentration, it tapers off towards x-axis. Thus for synthetic hemin, which has four phenyl moieties at 5, 10, 15 and 20 positions, shows that two molecules of heme are binding per molecule of protein.

The physico-chemical behaviour of the plasma albumin’s has been studied [1] in detail. The two important properties of plasma proteins, namely the ability to bind a very large variety of substances, both ionic and non-ionic, and the ability of the protein to undergo extensive reversible alterations in three dimensional folded structure. Probably the two types of phenomena were interrelated [1]. There was evidence that the single polypeptide chain might be folded into two similar globular units which in the native protein in the neutral solution were closely associated with one another. Probably the association was due mainly to the existence of largely hydrophobic surfaces which tend to associate as a means of escaping the aqueous environment (hydrophobic interaction). Since both the hemes are hydrophobic in natu-
Figure 5.2: Absorption spectra of tetraammonium tetra (p-sulfophenyl) porphine (TPPS₄) showing the typical 392 nm band. Inset: UV-Vis absorption band at 528 and 680 nm.
Figure 5.3: Fluorimetric titrations of bovine serum albumin with haemin in 5 mM tris buffer, pH 7.7, showing the stiochiometric ratio of binding. The binding is found to be 1:1-2 per role of BSA. The line is drawn only to show the nature of curvature.
re, it is conjectured that the binding site on the protein for the heme is probably same but large enough, to accommodate two synthetic heme molecules. But when, natural heme which is relatively small in size compared to FeTPPS₄ binds one molecule, the binding is relatively tight and other molecules with non-specific binding and affinity.

There are three types of quenching mechanisms by which the fluorescence data can be analysed. Static, Dynamic and the combined dynamic and static. Static quenching is as a result of formation of non-fluorescent complex between the fluorophore and the quencher; Dynamic quenching is due to time dependant diffusive collisions between the fluorescence 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 [17], chapter 2.

If both dynamic as well as static quenching processes are operative to varying degrees, the relative fluorescence is represented by:

\[
\frac{F_0}{F} = (1 + K_D [Q]) (1 + K_S [Q])
\]  

In figure 5.2, we have plotted relative fluorescence intensity \( \frac{F_0}{F} \), as a function of the ratio of quencher to protein. Therefore,

\[
\frac{F_0}{F} = 1 + (K_D + K_S) [Q] + K_D K_S [Q]^2
\]  

When dynamic quenching component is very small or identically zero, then equation (2) becomes;

\[
K_D = 0,
\]

\[
\frac{F}{F_0} = 1 + K_S [Q]
\]  

Also, equation (2) can be represented as,

\[
K_{app} = (K_D + K_S) + K_D K_S [Q] = \frac{F_0}{F} - 1
\]
Figure 5.4: Fluorimetric titrations of bovine serum albumin with FeTPPS$_4$ (synthetic haemin) in 5 mM tris buffer, pH = 7.7, showing the stoichiometric ratio of binding. The binding is found to be 1:2 per mole of BSA.
Figure 5. 4 shows the plot of relative fluorescence intensity as a function of quencher concentration for FeTPPS$_4$ interaction. The linearity shows that equation 3 is valid. Representing the data as \( \left( \frac{F_0}{F} - 1 \right) \) versus quencher concentration did not produce any change and the Stern-Volmer constant for dynamic quenching, \( K_D \) was found to be negligibly small, \( K_D = 0.07 \, \mu M^{-1} \) for both hemin as well as FeTPPS$_4$. The quenching of BSA with heme is definitely static and the Stern-Volmer constant for static quenching were deduced to be 23.8 \( \mu M^{-1} \) and 26.7 \( \mu M^{-1} \). These are fact, the association constants for heme and FeTPPS$_4$ respectively. The association constant of natural heme to human serum albumin (HSA), a structurally similar protein found in human blood has been reported to be 50\( \mu M^{-1} \) [17]. Thus affinity of natural heme for BSA is weaker as compared to HSA. However, it is seen that synthetic heme has comparable but share higher affinity for BSA than natural heme. This seems consistent with what we conjectured earlier, that natural heme has non-specific binding affinity for more than one molecule per BSA molecule, but FeTPPS$_4$ which shows almost quantitative binding ratio of 1:2 has correct orientation for binding.

The fact that FeTPPS$_4$ has almost the same association constant (that is the affinity) as that of natural heme, because FeTPPS$_4$ has four pyrrole rings, having four phenyl groups compared to the heme having none. The benzene rings are increasing the hydrophobicity and hence no change in the affinity of FeTPPS$_4$ to BSA is being experienced. So, the binding site in the protein is probably hydrophobic. The FeTPPS$_4$ is a big molecule and is also experiencing steric hindrance as well. With concentration a linear correlation was obtained with FeTPPS$_4$, meaning thereby that the synthetic heme is monomeric.

We concluded that the quenching by heme and synthetic heme to BSA is mostly static as the association constant was respectively as 23.8 and 26.7 \( \mu M \) although dynamic quenching component is also that, which is very very small as compared to static quenching but it is not zero. i.e., \( K_S \gg K_D \)

In terms of antibody-antigen correlation, we can discuss that the combining sites of antibodies i.e., their antigen-binding sites resembles the active sites of enzymes. The binding of heme or FeTPPS$_4$ quenches the fluorescence of the tryptophan residues of the antibody. The extent of quenching is the measure of the saturation of the antibody.
Figure 5.5: The figure shows the plot of relative fluorescence intensity as a function of quencher concentration for FeTPPS₄ interaction. The linearity shows the validity of equation (3). (see Text)
combining sites. Furthermore, the binding forces in heme or synthetic heme complexes are like those in enzyme-substrate complexes. Weak, non covalent interactions of the electrostatic, hydrogen bond, and Van der waals types combine to give strong and specific binding.
REFERENCES


