Spectrofluorimetric Investigation of Interaction of Coumarin-1 with Bovine Serum Albumin

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CERTIFICATE

This is to certify that the dissertation entitled **"Spectrofluorometric Investigation of Interaction of Coumarin1 with Bovine Serum Albumin"** being submitted by Miss Dipikapryidarsini Jena and Miss Juisa Nandini Patra to the Department of Chemistry, National Institute of Technology, Rourkela,Orissa, for the award of the degree of Master of Science is a record of bonafide research carried out by her under my supervision and guidance. To the best of my knowledge, the matter embodied in the dissertation has not been submitted to any other University / Institute for the award of any Degree or Diploma.

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ABSTRACT

Serum albumin is the most abundant protein in blood plasma. Many drugs, including anticoagulants, tranquilizers and general anesthetics are transported in the blood while bound to albumin. Coumarins constitute an imported group of natural products that show high binding affinity towards serum albumins. Binding of coumarin-1 (7-N,N-diethylamino-4-methylcoumarin) to bovine serum albumin (BSA) results in seven fold enhancement in its fluorescence intensity, 20 nm blue shift in emission maximum and ten fold increase in fluorescence anisotropy and around four fold increase in the fluorescence lifetime as compared to that in water indicating strong binding efficiency. It shows two binding sites in BSA determined by Job's plot, and one high affinity site with a larger binding constant (*ca*. 10^5 M^{-1}) and other low affinity site with a smaller binding constant (*ca*. 10^4 M^{-1}) evaluated from Scatchard plot. The Edsall plot and Bjerrum plot reveal the highly interactive nature of the two binding sites and the site specific ligand displacement experiments suggests the binding of Cou 1 to the warfarin binding site *i.e.* site I in subdomain IIA.

CHAPTER-1
INTRODUCTION

1.1 SERUM ALBUMIN:

Albumin, the most abundant extracellular protein accounts for total 60 % of the total serum content in mammal. It is manufactured in the liver and is a single polypeptide with 582 amino acids and a molecular weight of 66200 D for human serum albumin [1]. It is a highly soluble multi-domain protein, without prosthetic groups or bulky appending carbohydrates, which is very stable and available at high purity. Human serum albumin (HSA) structure has been revealed by high-resolution X-ray image of the protein [1, 2], which is directed towards the determination of the tertiary structure of other mammalian albumins as they resemble closely to it. There are more than 50 slight variants of the 582 amino acid sequences that comprises human albumin exists. The primary structure of serum albumin differs from other extracellular proteins. Serum albumin has one cysteine group (Cys-34) and low tryptophan content. The secondary structure consists of approximately 67 % of α-helix as well as there are 9 loops and 17 disulphide bridges giving a heart shaped 3D structure confirmed by X-ray crystallography studies [2, 3]. The tertiary structure is composed of three domains I, II, and III, and each domain is constituted of two subdomains A and B.

It can be described as a very flexible protein that changes shape with variations in environmental conditions and with binding of ligands. Despite this albumin has a resilient structure and regains shape easily owing to the disulphide bridges, which provides strength especially in physiological conditions. After their rupture the molecule can re-establish these bridges and regain its structure [4] encouraging its study for the better elucidation of structural and binding characteristics of the complex formed by the interaction with different drugs for the pharmaceutical applications.

1.1.1Function of serum albumin:

Serum albumin has been one of the most extensively studied being the most abundant protein in blood plasma with typical concentration of 50 g/L. Some of the albumins most commonly studied are human serum albumin (HSA), bovine serum albumin (BSA), equine serum albumin (ESA) and rat serum albumin (RSA).

Physiological Roles of albumin:

1. Maintenance of the colloid osmotic pressure (COP)

   Albumin is responsible for the 75-80 % of osmotic pressure in blood. It constitutes the main protein in the blood plasma and in the interstitial fluid. It defines the flow of fluid in and out of the capillaries [4].

2. Binding and transport, particularly of drugs

   Albumin helps in transport of drugs and ligands by binding to it and reduces the serum concentration of these compounds. There are particularly four binding sites on albumin with varying specificity for different substances. Competitive binding of drugs may occur at same site or different sites leading to conformational changes, for example: warfarin and diazepam. In other words they can be considered as carriers for numerous exogenous and endogenous compounds in the blood [2, 3].

3. Free radical scavenging

   Albumin is a major source of sulfhydryl groups, these "thiols" scavenge free radicals (nitrogen and oxygen species) [4].

4. Acid base balance

   Albumin is a negatively charged protein in high concentration in the plasma. It contributes heavily to what is called as the “anion gap”: Classically the anion gap is calculated as (Na + K) − (Cl) = AG (mEq/l). The concentration of anions and cations in plasma should be equal, so the remaining anions come predominantly from albumin, inorganic phosphate and haemoglobin [4].

5. Effects on vascular permeability

   Albumin has a role in limiting the leakage from capillary beds during stress induced increase in the capillary permeability. This is related to the ability of endothelial cells to control the permeability of their walls, and the spaces between them. Albumin may plug this gap or may have a deflecting effect owing to its negative charge. This has led to the hypothesis that colloids are effective at maintaining vascular architecture [4].

1.1.2 Structure of Bovine Serum Albumin (BSA):

The primary structure of BSA was proposed after HSA structure elucidation and is composed of 585 amino acid residues. The sequence has 17 disulphide bonds resulting in nine loops formed by the bridges. BSA contains one cysteine and 8 pairs of disulphide bonds similar to HSA [1]. BSA also contains a high content of Asp, Glu, Ala, Leu and Lys as well as the four amino acid residues in the sequence determined later as Gly–Phe–Gln–Asn [5].

According to the amino acid sequence proposed by Brown, the structural features of BSA show that it is composed of three homologous domains [5, 6]. Circular dichroism measurements suggest that BSA secondary structure content for α- helix, β- sheet, turn and random coil are 48.7%, 0%, 10.9% and 30.7%, respectively [7, 8]. In the secondary structure of BSA, it has been suggested that the α-helices are uniformly placed in the subdomains and in the connections between the domains. Most of the residues in the long loops (except at the end) and the sections linking the domains possibly form α-helices, whereas the intra-domain hinge regions are mainly non-helical structure. The three long helices in the subdomain are considered as principle elements of the structure. These run parallel with each other, and a trough is formed owing to the middle helix (Y) being slightly lower in position. The helices are mainly linked together by disulphide bridges [3].

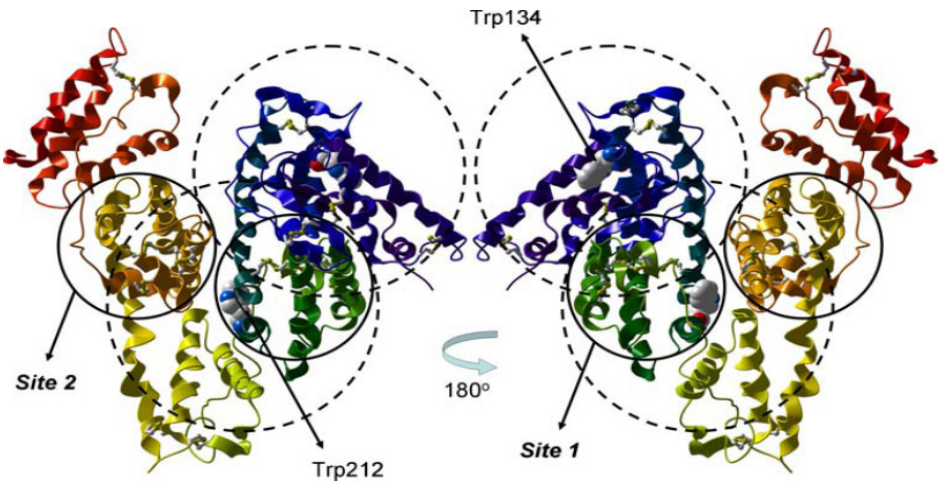

Fig1.1: Two side-on 3D graphic representation of a BSA model structure based on HSA X-ray crystal structure obtained from the Protein Data Bank (PDB ID:1UOR) [1, 2].

The 3D graphic structure (Fig. 1.1) is in accordance with the proposed domains and sub-domains present in the BSA. It shows clearly the presence of two tryptophan residues that is basically responsible for the intrinsic fluorescence of BSA.

1.1.3 Difference between HSA and BSA:

HSA and BSA are the most studied serum albumin proteins. There occurs almost 76% homology and a repeating pattern of disulphides which is conserved. The major difference between the two occurs with respect to the number and positioning of tryptophan residues in them. HSA has only one tryptophan, located at position 214, which is equivalent to Trp-212 for BSA buried in a hydrophobic pocket at sub domain IIA. BSA has one more additional tryptophan Trp-134, which is more exposed to solvent and found at sub domain IB [2, 4]. Thus BSA which is a homologous protein of HSA is selected as the protein model due to its medical importance, low cost, ready availability, and unusual ligand-binding properties [1, 8-9].

1.2 Fluorophore

A fluorescent molecule or otherwise called fluorophore has the ability to absorb photons of energy at one wavelength and subsequently emit the energy at another wavelength. The absorption process is also called excitation, since the quantum energy levels of some of the compound's electrons increase with photon uptake. The extinction coefficient (ε, expressed as $M^{-1}cm^{-1}$) at the absorbance peak maximum is a unique characteristic of each fluorophore under a given environmental condition. The maximum wavelength of absorption (excitation) and emission are important specifications to define the fluorophore. Fluorescence spectroscopy of proteins as a methodology is widely used to yield the structural and dynamical information concerning the fluorophore microenvironment of the macromolecule in study. It is basically of two types: intrinsic fluorophore and extrinsic fluorophore.

1.2.1 Intrinsic Fluorescence

Protein contains three amino acids residues namely Tryptophan, Phenylalanine, Tyrosine which contribute to the intrinsic fluorescence. The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues.

Protein fluorescence is observed generally by exciting at 280 nm or at higher wavelengths usually at 295 nm. The emissions are due to the excitations of tryptophan residue predominantly when excited at 295 nm and a combination with contributions from the

tryptophan, tyrosine and phenylalanine residues when excited at 280 nm. These three residues have distinct absorption and emissions wavelengths. They differ greatly in their quantum yields and lifetimes. Due to these differences and due to resonance energy transfer from proximal phenylalanine to tyrosine and from tyrosine to tryptophan the fluorescence spectrum of a protein containing these residues usually resembles that of tryptophan.

Table 1.1: Fluorescent Characteristics of the Aromatic Amino Acids. [10]

	Absorption		**Fluorescence**	
Amino Acid	**Wavelength (nm)**	**Absorptivity ($M^{-1}cm^{-1}$)**	**Wavelength (nm)**	**Quantum Yield**
Tryptophan	280	5,600	348	0.20
Tyrosine	274	1,400	303	0.14
Phenylalanine	257	200	282	0.04

Tryptophan has much stronger fluorescence and higher quantum yield than the other two aromatic amino acids. The intensity, quantum yield, and wavelength of maximum fluorescence emission of tryptophan are very much solvent dependent. The fluorescence spectrum shifts to shorter wavelength and the intensity of the fluorescence increases as the polarity of the solvent surrounding the tryptophan residue decreases. Tryptophan residues that are buried in the hydrophobic core of proteins can have spectra, which are blue or red shifted depending on the polarity experienced by them due to the solvent.

Tyrosine, like tryptophan, has strong absorption bands at 280 nm, and when excited by light at this wavelength, has characteristic emission. Tyrosine is a weaker emitter than tryptophan, but it may still contribute significantly to protein fluorescence because it is present in larger numbers. The fluorescence from tyrosine can be easily quenched by nearby tryptophan residues because of energy transfer effects.

Phenylalanine with only a benzene ring and a methylene group is weakly fluorescent. The quantum yield and molar absorptivity is low for this residue. Phenylalanine fluorescence is observed only in the absence of both tyrosine and tryptophan.

1.2.2 Extrinsic Fluorescence

Extrinsic fluorescence probes are relatively small molecules compared to the macromolecules like proteins, nucleic acids, and other molecules used during the study. These compounds often contain groups that provide sensitive detectability by virtue of some intrinsic chemical or atomic property as fluorescence towards protein. These fluorescent external probes during study of macromolecules result in either quenching of macromolecule fluorescence or else the remarkable increase of fluorescence of the probe on binding to macromolecule depending on the nature of the probe.

Some of the commonly used fluorophores in the spectroscopic study of proteins to mention are Pyrene, ANS, Nile blue, Nile red and Fluorescein *etc.* [11-16].

1.3 Importance of binding analysis study

Albumins have been identified as major transport proteins in blood plasma for many compounds such as fatty acids, hormones, bilirubin and many drugs which are otherwise insoluble in plasma [17]. Serum albumins are thus effective in increasing the solubility of hydrophobic drugs in plasma and modulate their delivery to cell *in vivo* and *in vitro*. They also play a leading role in drug disposition and efficacy. Furthermore, albumins are the principal biomacromolecules that are involved in the maintenance of colloid-blood pressure and are implicated in the facilitated transfer of many ligands across organ-circulatory interfaces such as in the liver, intestine, kidney, and brain [18]. From a biopharmaceutical point of view, one of the most important biological functions of albumins is their ability to carry drugs as well as endogenous and exogenous substances, and numerous experiments with the aim of characterizing the binding capacity and sites of albumins have been carried out [19-21]. The drugs move through the blood by binding with the serum albumins only, so it is necessary to account for their interaction to obtain the needed best results.

Understanding the full implications of the human genome project and the emerging field of proteomics has introduced new needs to further probe millions of protein interactions. Analysis is based on monitoring the change of a physicochemical property of the protein–probe system upon binding either directly (direct technique) or after separation of the bound and free probe (indirect technique). Among the direct techniques, fluorescence analysis is extensively used and considered to be superior to the indirect techniques (equilibrium and dynamic dialysis, ultrafiltration and gel filtration) because, it does not disturb the binding equilibrium upon separation [22]. The spectral changes observed on the binding of fluorophores with proteins are an important tool for the investigations of the topology of binding sites, conformational changes and characterization of substrate to ligand binding [23-

25]. Besides, determination of protein quantity in biological liquids is of great importance in biology and medicine and fluorescent probes are successfully applied for this approach. The capability of serum albumins to bind aromatic and heterocyclic compoundsis largely dependent on the existence of two major binding regions, namely Sudlow's site I and site II, [2] which are located within specialized cavities in subdomains IIA and IIIA, respectively. These hydrophobic binding pockets enable the serum albumins to increase the apparent solubility of the hydrophobic drugs in plasma and modulate their delivery to the cells in vivo and in vitro. Many drugs, including anticoagulants, tranquilizers, and general anaesthetics, are transported in blood while bound to albumin-often more than 90% of the drug is bound [26, 27]. This has stimulated much research on the nature of drug binding sites as well as investigations on whether fatty acids, natural metabolites, and drugs compete with each other for binding to the protein [28-35]. Various techniques, including fluorimetry [36- 40], equilibrium dialysis [41], circular dichroism spectroscopy [42, 43], UV spectrophotometry [44- 46] and electrochemistry [47, 48], FTIR [49, 50] and NMR [51] are usually used for studying molecular interaction of drugs with SA. Among these methods, fluorescence spectroscopy has been particularly useful with the application of site marker fluorescence probes for investigating the nature of the binding sites as well as their specificity and affinity towards particular ligands.

A quantitative analysis of the binding interaction is necessary to study the drug to protein interaction. From *in vitro* experiments that analyze the binding process, a number of parameters can be determined such as:

(i) How many ligands bind per protein molecule (the stoichiometry of binding),

(ii) The binding affinity or equilibrium binding constants for a given site as determined by the sequence,

(iii) The specificity of the binding interaction, which can be deduced from comparison with the binding affinity

(iv) The effect of already bound ligand on the binding of additional free ligand (cooperativity of binding) [52].

Nitin Chattopadhyay *et al.* have reported the photophysical study on the binding interaction of an efficient polarity sensitive photosensitizer norharmane with BSA and HSA using time

resolved and steady state fluorescence techniques [53]. Soumen Ghosh *et a.l* have studied the surfactant ATAB, C12ES and SDS with BSA to ascertain the binding site, binding constant as well as the denaturation study [54]. Nikhil Guchhait *et al.* have recently reported the interaction of an ICT compound DPDAME for spectroscopic probing of BSA and to evaluate the stability in presence of denaturant SDS and urea with determination of binding characteristics in each case [55]. The binding characteristics of wogonin with BSA was investigated at different temperature by fluorescence, CD, FTIR by Jianniao Tian *et al.* [56] and Xianyong Yu *et al.* investigated interaction of salvianic acid A sodium interaction with BSA [57]. Recently Suresh Kumar *et al.* have investigated the binding analysis of phototoxic alkaloid coralyne by UV and fluorescence techniques [58]. Coumarin 153 interaction with BSA has been studied by Nilmoni Sarkar *et al.* using ANS as a standard probe accompanied by denaturation studies with SDS to evaluate the binding characteristics [59].

1.3.1 Different techniques used for study of binding process:

The binding process can be followed using a lot of different experimental approaches. Fluorescence spectroscopy is a reliable tool in the study of proteins due to its great sensitivity and selectivity. In many cases, the protein of interest will contain one or more amino acids that are intrinsically fluorescent (phenylalanine, tyrosine, or tryptophan). The characteristics of these fluorescent amino acids have been clearly described in results and discussion part to account for the changes during the interaction process. Extrinsic fluorophores attached to the protein can also be used to extend the possible range of protein binding studies in particular where an intrinsic fluorescent amino acid is absent or not sufficient to account for the same. Geometrical effects can be successfully quantified using Forster resonance energy transfer (FRET) where the changes in distance between donor and acceptor centres can be monitored [56]. The other techniques used are circular dichroism, dynamic light scattering, UV-Visible spectroscopy and many others to study the binding process of proteins depending on the probe used.

1.3.2 Binding sites of albumin

In the 1970s, two main drug-binding sites were proposed in HSA, denoted as Sudlow site I and Sudlow site II. They are located in the hydrophobic cavities of subdomains IIA and IIIA, respectively. Sudlow site I was shown to prefer large heterocyclic and negatively charged compounds (Structure 1), while Sudlow site II was the preferred site for small aromatic

carboxylic acids [60]. Other highly specific binding sites include one for binding polycyclic aromatic hydrocarbon epoxides in subdomain IB, and as many as six for fatty acids [10]. In addition, there are a number of minor binding sites that allow multiple drug molecules to bind simultaneously onto HSA [55]. BSA demonstrates the highest degree of similarity to HSA in terms of tertiary structure and binding characteristics. The main structural difference between BSA and HSA pertains to the distance between subdomains IIA and IIB as well as the number of tryptophan present, which is of interest for intrinsic protein fluorescence.

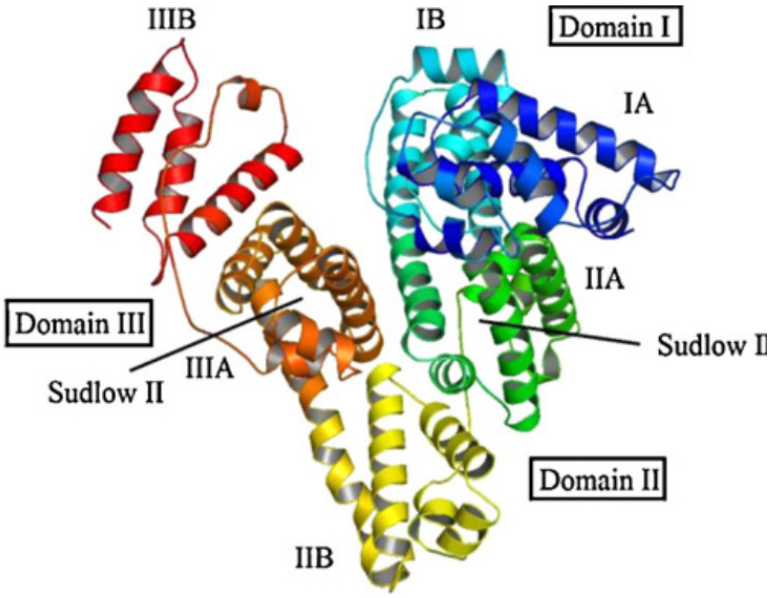

Fig: 1.2 Pictorial Representation of drug binding sites In BSA [61]

1.3.3 Drugs binding to particular binding sites of Human Serum Albumin [3]

Table 1.2: List of potential drugs binding to particular sites in HSA.

Compound	**Association constant, K (M^{-1})**	**n (No. of moles of ligands per mole of protein)**	**Percent free**
Site I			
Salicylate	1.9×10^5	1	16
Sulfisoxazole	1.8×10^5	1	15
Warfarin, S-(—)	3.3×10^5	1	3

Phenylbutazone	7.0×10^5	1	1
Indomethacin	1.4×10^6	1	1
Tolbutamide	4×10^4	1.4	1
Furosemide	2.6×10^4	1.6	3
Phenytoin	6×10^3	6	9
Chlorpropamide	3.3×10^5	1	4
Chlorthiazide	3.1×10^4	2	11
Oxacillin	4.7×10^3	1	6
Benzylpenicillin	1.2×10^3	1	75
Acetotrizoate	4×10^4	1	-
Phenol red	2.8×10^4	1	-
Bromcresol green	7×10^5	3	-
Bromphenol blue	1.5×10^6	3	-
Iophenoxate	8×10^7	-	-
Sulfobromophthalein	1.7×10^7	-	-
Methyl orange	2.2×10^3	-	-
Methyl red	2.2×10^5	-	-
Evans blue	4.0×10^5	14	-
Site II			
Diazepam (S)	3.8×10^5	1	1
Ibuprofen	2.7×10^6	1	-
Octanoate	5.5×10^5	1	-
Clofibrate	7.6×10^5	1	10
Site III			
Digitoxin	0.4×10^5	1	3
Site I and Site II		-	-
Naproxen	1.2×10^6	1	<1

1.4 AIM OF THE PRESENT WORK:

Coumarins constitute an important group of natural products and many of their analogues are found to be biologically active. Coumarin 1 (7-N,N-diethylamino-4-methylcoumarin), a 7-

aminocoumarin, belongs to the class of ICT molecules. Coumarin 1 shows structural similarity with warfarin, so was thought to be explored as a new probe which can overcome the relative interference from the excitation wavelength of warfarin (320 nm) which is close to intrinsic tryptophan wavelength of protein (295 nm) whereas of Coumarin1 (370 nm) is widely separated. This provides the liberty to monitor both the probe and protein fluorescence.

CHAPTER-2
MATERIALS AND METHODS

2.1 MATERIALS:

2.1.1: Protein used:

Bovine serum albumin fraction V (BSA- protease free) was purchased from SRL India and used as such.

2.1.2: Extrinsic Probe used:

Coumarin 1 was obtained from Sigma Aldrich and used after recrystallizing with ethanol. Warfarin needed for site specific determination was obtained from Sigma Aldrich and used as such.

2.1.3 Solvent Used :

Deionised water was used for all the experiments. All the solvents used were of spectroscopic grade (SRL India Pvt. Ltd.) and used as received.

2.1.4 Instrumentation:

The absorption spectra were recorded using ***Shimadzu Spectrophotometer (UV-1800)*** and the emission spectra and steady-state fluorescence anisotropy values were recorded using ***Horiba Jobin-Yvon Spectrofluorimeter (Fluoromax-4P).***

2.2 METHODS:

2.2.1 Preparation of Solutions for Fluorescence Measurements:

The BSA stock as required was prepared in deionised water freshly every time. The studies were carried out by giving an equilibration time of about 30 minutes. The stock solutions of Coumarin1 (10^{-3} M) was prepared in methanol. Then the dilution was done with water to obtain the required concentration of experimental solution each time. The concentration of methanol was maintained less than 25 mM so that it does not alter the binding of fluorophore as well as does not affect the protein structure.

2.3 PARAMETERS STUDIED:

Fluorescence intensity of Coumarin 1 as well as Tryptophan depends upon the polarity of the medium.

Steady State Fluorescence anisotropy 'r' is dependent on the rigidity of the microenvironment and the rotational mobility of the fluorophore and is defined as

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

G is the ratio of the sensitivities of the detection system for parallel and perpendicular light. Generally the value is 1. The high value of anisotropy suggests the rigidity of microenvironment as well as restricted mobility of fluorophore whereas low value corresponds to the free mobility of fluorophore.

2.4 METHODS OF DATA ANALYSIS:

2.4.1 Data Analysis

Data analysis was done using Microcal Origin 7 software.

2.4.2 Job's Plot[62]

Job's plot is a technique used to characterize the composition of the complex formed. In the present case, Job's plot was constructed by monitoring the fluorescence intensity change of Coumarin 1 at 435 nm upon binding to protein. Solutions of BSA and Coumarin 1 of equal concentration were prepared and they were mixed together in different volume ratios so as to have constant total concentration. The presence of a maximum or a minimum in the plot of mole fraction versus the observable property gives information about the stoichiometry of the probe -protein complex [62].

2.4.3 Binding Constant Studies [63, 64]

It is otherwise known as fluorescence-used determination of binding constant

Let the fraction of bound probe is X.

X is determined by,

$$X = \frac{F_p - F_0}{F_b - F_0}$$

Where F_p, F_0, F_b are the fluorescence intensity of a given concentration of the probe in a solution of low protein concentration, in a solution without protein and of fully bound probe, respectively [73].

Concentration of bound probe [B] is given by,

[B] = the fraction of bound probe × concentration of probe

And [D] = Total concentration − [B]

Where [D] is the free probe concentration.

2.4.4 Scatchard Plot [63, 64]

After we get the value of the fraction of the bound probe determined for all the points along the titration curve, the number of moles of probe bound per mole of protein was determined using

$$\bar{n} = \frac{[B]}{[\text{Protein conc.}]}$$

the results were then plotted according to Scatchard equation given as

$$\frac{\bar{n}}{D} = NK - \bar{n}K$$

Where "$\bar{n}$" is the number of moles of Coumarin 1 bound per mole of protein,

N is the maximum number of binding sites,

K is the binding constant.

The plot of $\frac{\bar{n}}{[D]}$ vs $\bar{n}$ gives the binding constant, K.

2.4.5 Edsall Plot [65, 66]

Scatchard plot assumes that the sites are identical and non- interacting. Edsall's treatment involves the use of a modified Scatchard equation to account for the interacting or non-interacting nature of binding sites. The equation is as follows:

$$\log Q = \log\left[\frac{\bar{n}}{N - \bar{n}}\right] - \log[D]$$

where Q is a function of the equilibrium constant, $\bar{n}$ equals the number of moles of substrate bound per mole of protein, N equals the maximum number of binding sites, and [D] equals the equilibrium concentration of free substrate. Plots of log Q vs. $\bar{n}$ are constructed with trial values of N to access the nature of binding sites. If the sites were identical and non-interacting then function Q should be a constant independent of $\bar{n}$ and slope of the plot of log Q vs. $\bar{n}$ will be a straight line with slope equal to zero. But if the sites are interaction log Q will be a linear function of $\bar{n}$ with non-zero slope.

2.4.6 Bjerrum Plot [65, 67]

A binding constant is an equilibrium constant for the formation of a complex in solution. It is a measure of the strength of the interaction between the reagents (extrinsic probe and protein) that come together to form the complex (protein - probe). The simultaneous equilibria have to be considered for the formulation of the equation for the binding constant determination which has been described in detail in the results and discussion.

2.4.7 Benesi-Hildebrand Treatment [55, 68]

The Benesi-Hildebrand method is a mathematical approach used in physical chemistry for the determination of the equilibrium constant K and stoichiometry of non-bonding interactions. This method has been typically applied to reaction equilibria that form one-to-one complexes, such as charge-transfer complexes and host-guest molecular complexation which assumes 1:1 binding and with one component present in excess to the other. The changes monitored during absorption and also fluorescence are used to obtain the binding constants through the non linear regression fit to the characteristic Benesi- Hindebrand equation depending on the property monitored.

2.4.8 Site I Specific Ligand Replacement Study [53-60]

The site displacement experiments were performed using the site I specific probe warfarin keeping the BSA and the Warfarin concentrations at 1:1 with [BSA] =2.5 x 10^{-5} M, [Warfarin] = 2.5 x 10^{-5} M and varying the Cou 1 concentration between 0 M - 16 x 10^{-5} M. The fluorescence emission spectra of warfarin and Cou 1 were monitored by exciting at 320 nm and 370 nm, respectively.

CONCLUSIONS

- Remarkable enhancement in fluorescence intensity, steady state fluorescence anisotropy and average fluorescence lifetime and appreciable blue shift in the emission maximum of Cou 1 indicates a strong binding of Cou 1 to BSA.

- The stiochiometry of binding was determined to be 1:2 for protein – Cou 1 complex from Job's plot.

- One high affinity site with a larger binding constant (*ca.* 10^5 M^{-1}) and other low affinity site with a smaller binding constant (*ca.* 10^4 M^{-1}) was obtained from the Scatchard analysis.

- The Edsalls and Bjerrum plot analysis confirms multiple binding with interacting and closely associated equilibrium of binding of BSA – Cou 1.

- The binding constant obtained from Benesi- Hildebrand is the average of the high and low affinity site binding constants assuming 1:1 complexation at low concentration of Cou 1.

- The site specific ligand replacement studies with warfarin suggests the binding of Cou 1 to site 1 of BSA.

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