binding of bioactive coomassie brilliant blue G with protein:
insights from spectroscopic studies

abstract

The binding of \textbf{CBB} to \textbf{BSA} was investigated under imitated physiological conditions employing different optical spectroscopic techniques \textit{viz.}, fluorescence emission, UV-vis absorption and FTIR. Fluorescence quenching data obtained at different temperatures suggested the presence of dynamic type of quenching mechanism. The values of $K$ and $n$ for CBB-BSA system were calculated and found to be $4.20 \times 10^4 \text{ M}^{-1}$ and 0.96 respectively, at 302 K. The value of $n$ close to unity indicated that one molecule of CBB bound to one molecule of BSA. The thermodynamic parameters revealed that the hydrophobic forces played a major role in the interaction of CBB with BSA. The distance of separation between CBB and protein was calculated using the theory of FRET. The conformational changes in the secondary structure of BSA upon interaction with the dye were investigated by synchronous fluorescence and FTIR techniques. Competitive binding studies were also carried out to locate the binding site of CBB on BSA.

The results of this chapter are published in \textit{Scientia Pharmaceutica, 78} (2010) 869-880.
## Coomassie brilliant blue-G (CBB)

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Benzenemethanaminium, N-[4-[[4-[(4-ethoxyphenyl)amino]phenyl][4-[ethyl[(3-sulfanyl)methyl]amino]phenyl]methylene]-2,5-cyclohexadiene-1-ylidene]-ethyl-3-sulfo-monosodium salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Molecular formula</td>
<td>$C_{47}H_{48}N_{3}O_{7}S_{2}Na$</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>854.02</td>
</tr>
<tr>
<td>Description</td>
<td>Purple coloured solid</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in water and in common organic solvents</td>
</tr>
<tr>
<td>Category</td>
<td>Purinergic receptor antagonist dye</td>
</tr>
</tbody>
</table>
INTRODUCTION

**CBB** dye (also known as coomassie dye) is employed to stain proteins in sodium dodecyl sulfate and blue native polyacrylamide gel in electrophorosis gels. It is used in the quantification of electrophoretically separated protein [1]. The CBB has also been popularly used in biochemical and clinical laboratories for the purification and quantification of proteins [2].

**SA** is essential for the maintenance of osmotic pressure which is needed for proper distribution of body fluids between intravascular components and body tissues. SA consists of three homologous domains (I-III), each of them is composed of two subdomains (A and B) [3, 4]. In IIA and IIIA pocket, the majority of small ligands like drugs are bound and carried to the target site [5-8]. Therefore, it is important to study the interaction of the drug with SA, which plays an important role in the field of pharmacology, pharmacokinetics and pharmacodynamics. In this regard, **BSA** has been studied extensively, partly because of its structural homology with HSA [9].

In the present work, we have focused on the binding of CBB to BSA and obtained the information with regard to binding constant, number of molecules of binding, thermodynamic parameters and conformational changes in the protein employing spectroscopic techniques. Fluorescence technique is a well known practical method for studying protein interactions with various ligands [10-12] as it gives a vast amount of information on binding characteristics and the microenvironment surrounding the protein.

**EXPERIMENTAL**

**Reagents**

The solutions of CBB and BSA were prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl.

**Apparatus**

The details of instruments employed in the present study are given on page number 22.
Procedures

Fluorometric studies

The fluorometric titrations were carried out and the fluorescence intensities of protein were recorded at around 340 nm upon excitation at 296 nm. Based on preliminary studies, the concentration of BSA was fixed at 2.5 μM while that of the dye was varied from 0 to 25 μM. The interaction studies were carried out at different temperatures viz., 293, 302 and 309 K.

Distance of separation between CBB and BSA

The overlap of the emission spectrum of BSA (2.5 μM) and the absorption spectrum of CBB (2.5 μM) was used to calculate the distance of separation between CBB and BSA on the basis of theory of fluorescence resonance.

Synchronous spectral studies

Synchronous fluorescence spectra of BSA in the absence and presence of different amounts of CBB were obtained by simultaneously scanning the excitation and emission monochromater maintaining $\Delta \lambda = 15$ nm and $\Delta \lambda = 60$ nm between them. These experiments were carried out at 302 K.

Absorption studies

The absorption spectra for CBB-BSA system were recorded by scanning the binary mixture of dye and the protein in the wavelength range of 250-350 nm at 302 K.

FTIR spectral studies

The FTIR spectra of BSA in the absence and presence of CBB were obtained in the range of 1500 to 1700 cm$^{-1}$ using the procedure shown on page number 30.
**Displacement experiments**

The site probe studies were performed using different probes for site I, II and III by keeping the concentration of both protein and the probe, constant (each at 2.5 μM) and varying the concentration of the dye from 0 to 25 μM.

**RESULTS AND DISCUSSION**

**CBB-induced quenching of BSA**

Fluorescence spectra of BSA were recorded in the presence and absence of CBB upon excitation at 296 nm. The fluorescence emission of protein was observed to be quenched (~ 340 nm) in a concentration dependent manner by CBB (Fig. 1). Quenching of the intrinsic fluorescence of protein was used to retrieve information on ligand-protein binding. The fluorescence quenching data were analyzed using the Stern-Volmer equation shown on page number 32.

The plot of the fluorescence intensity ratio of BSA in the absence and presence of quencher (CBB) as a function of the quencher concentration showed linear dependence (Fig. 2). The values of $K_{SV}$ for CBB-BSA system were calculated from the slope of the Stern-Volmer plot and the corresponding values are given in Table 1.

From the results of Stern-Volmer plots (Fig. 2) we have noticed that $K_{SV}$ values increased with increase in temperature indicating the presence of dynamic quenching mechanism in the interaction of CBB with protein [13]. The blue shift observed for $\lambda_{em}$ of CBB-BSA system revealed that the chromophores of the protein were placed in a more hydrophobic environment upon the addition of CBB. Moreover, we observed a concomitant increase in the fluorescence intensity at 401 nm, which is the characteristic wavelength of the bound BSA. This phenomenon might be the result of the radiationless energy transfer between BSA and CBB. Further, the existence of an isoactinic point at 375 nm indicated the presence of bound and unbound CBB at equilibrium.
**Determination of binding parameters**

CBB induced fluorescence quenching data of BSA were analyzed to obtain the binding parameters like $K$ and $n$ from the equation given on page number 33. **Fig. 3** depicts the linear plot of $\log \frac{(F_0-F)}{F}$ vs $\log [Q]$ and the corresponding results of $K$ and $n$ are given in **Table 1**. The values of $K$ were found to be increased with increase in temperature. The slope registered in this plot was noticed to be close to unity revealing that one CBB molecule bound to one molecule of protein. In other words, BSA has a single class of binding site for CBB. Hence, we have proposed that the CBB most likely bound to the hydrophobic pocket located in subdomain IIA, that is to say Trp-214 was near or within the binding site.

**Interacting force between CBB and protein**

There are essentially non-covalent interactions that play between the ligand and BSA. The signs and magnitudes of thermodynamic parameters determine the nature of interacting forces which are taking part in drug-protein interactions [14]. The values of $\Delta H^0$ and $\Delta S^0$ were calculated using the van’t Hoff’s equation shown on page number 34. The values of $\Delta G^0$ for the binding process at different temperatures were calculated using the equation shown on page number 34 and the corresponding results are given in **Table 1**.

The negative values of $\Delta G^0$ indicated spontaneity of the binding process. The positive values of both $\Delta H^0$ and $\Delta S^0$ observed in CBB-BSA system revealed that the hydrophobic forces stabilized the interaction between CBB and BSA [14].

**Transfer of energy between protein and dye**

The fluorescence quenching of BSA by CBB revealed the occurrence of energy transfer between the protein and dye. The efficiency, $E$ of a FRET process depends on the inverse sixth-distance between donor and acceptor ($r$) as well as on the critical energy transfer distance or Förster radius ($R_0$) under
the condition of 1:1 situation of donor to acceptor concentrations and can be expressed by using the equation given on page number 35.

**Fig. 4** depicts the overlap between the emission spectrum of free protein and the absorption spectrum of dye. In order to evaluate the Förster’s critical distance, the equation 8 shown on page number 35 was employed. Further, we have calculated the values of \( E, r, R_0 \) and \( J \) and found to be 0.28, 1.9 nm, 1.63 nm and \( 7.15 \times 10^{-16} \text{ cm}^3 \text{ m}^{-1} \) for CBB-BSA system, respectively. In the present study, \( r \) was considered as the average distance between the bound ligand and Trp residues [15]. The value of \( r \) less than 8 nm indicated the non-radiative energy transfer between BSA and CBB [13].

**Effect of CBB on the conformation of protein**

**Synchronous fluorescence measurements**

Synchronous fluorescence measurements were carried out in order to get the information on molecular environment in the vicinity of the fluorophores (Tyr and Trp) of protein. Synchronous fluorescence spectra of BSA (**Fig. 5**) were obtained by simultaneously scanning the excitation and emission monochromater maintaining \( \Delta \lambda = 15 \text{ nm} \) (for Tyr excitation) and \( \Delta \lambda = 60 \text{ nm} \) (for Trp excitation) between them. **Fig. 5** shows the effect of CBB on synchronous spectrum of protein when \( \Delta \lambda = 15 \text{ nm} \) (**Fig. 5a**) or \( \Delta \lambda = 60 \text{ nm} \) (**Fig. 5b**). It was evident that the intensity of Trp and Tyr decreased in the presence of CBB; but no significant shift was noticed in the signals. This indicated that the binding between CBB and the protein did not lead to a change in the polarity of the microenvironment of the Trp and Tyr residues; however, the internal packing of the protein was changed.

**UV absorption measurements**

We have recorded the absorption spectra of BSA in the absence and presence of CBB (**Fig. 6**). The absorption peaks observed around 280 nm were noticed to be shifted to the lower wavelength region with increase in the
concentration of CBB indicating the extension of the peptide strands of protein molecules [16, 17].

**FTIR spectral studies**

FTIR spectroscopy is a well defined tool for the determination of protein’s secondary structure [18, 19]. The conformational sensitivity of amide bands is governed by two most important factors viz., hydrogen bonding and the coupling between the transition dipoles. Both the amide I and amide II bands of the protein have the relationship with secondary structure of protein [20]. It was evident from Fig. 7a and 7b that the secondary structure of BSA was changed. The amide I band was noticed to be shifted from 1650.8 cm\(^{-1}\) (in free BSA) to 1646.9 cm\(^{-1}\) (in CBB-BSA) while amide II band was shifted from 1548.1 cm\(^{-1}\) (in free BSA) to 1554.6 cm\(^{-1}\) (in CBB-BSA complex).

**Location of binding site**

The principal regions of the ligand binding sites of albumin are located in the hydrophobic cavities of subdomain IIA and IIIA. In order to locate the binding site in BSA, we have recorded the fluorescence intensity of CBB-BSA in the presence and absence of site probe and in turn calculated the values of binding constant. The corresponding values are given in Table 2. It is clear from Table 2 that there was a significant decrease in the binding constant of CBB-BSA in the presence of warfarin. However, the binding constant values remained almost same in the presence of ibuprofen and digitoxin. These results revealed that the site I was the main binding site for CBB on protein. Therefore, the site I located in the hydrophobic pocket of subdomain IIA was proposed to be the binding site for CBB in protein.

**CONCLUSIONS**

The interaction between CBB and BSA was investigated for the first time by fluorescence spectroscopy, UV-vis absorption and FTIR spectroscopy under simulative physiological conditions. The fluorescence data indicated the strong
interaction as characterized by high binding constant value of $4.20 \times 10^4 \text{ M}^{-1}$ at 302 K. Further, the fluorescence quenching mechanism was observed to be dynamic process. Hydrophobic forces played as major force in the interaction process.
REFERENCES

**Fig. 1.** Fluorescence spectra of CBB-BSA system. Concentration of BSA was fixed at 2.5 μM (1) and that of CBB was varied in the range of 0-25 μM (2-11).

**Fig. 2.** The Stern-Volmer plot for quenching of BSA.
**Fig. 3.** The plot of $\log \left(\frac{F_0 - F}{F}\right)$ vs $\log [Q]$ for quenching of BSA by CBB at different temperatures.

**Fig. 4.** The overlap of fluorescence spectrum of BSA (1) and absorption spectrum of CBB (2); $[\text{BSA}] : [\text{CBB}] = 1 : 1$. 
Fig. 5. Synchronous fluorescence spectra of BSA: (a) $\Delta \lambda = 15$ nm; (b) $\Delta \lambda = 60$ nm. $C_{\text{BSA}} = 2.5$ μM (1), $C_{\text{CBB}} = 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0$ and $22.5$ μM (2-10).
Fig. 6. UV-vis absorption spectra of BSA in the absence and presence of various amounts of CBB; (1) $C_{\text{BSA}} = 2.5 \ \mu\text{M}$ (1) and $C_{\text{CBB}} = 0-25.0 \ \mu\text{M}$ (2-6). A concentration of $2.5 \ \mu\text{M}$ CBB (x) was used for CBB only.

Fig. 7. FTIR spectra and difference spectra of BSA: (a) free BSA (subtracting the absorption of the buffer solution from the spectrum of the protein solution) and (b) the difference spectra of BSA (subtracting the absorption of the CBB-free form from that of CBB-BSA bound form); $C_{\text{BSA}} = C_{\text{CBB}} = 2.5 \ \mu\text{M}$. 
Table 1.
Stern-Volmer quenching, binding and thermodynamic parameters of CBB-BSA system.

<table>
<thead>
<tr>
<th>Temp. (K)</th>
<th>K&lt;sub&gt;SV&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K (M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>n</th>
<th>ΔG&lt;sup&gt;0&lt;/sup&gt; (kJ mol&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>ΔH&lt;sup&gt;0&lt;/sup&gt; (kJ mol&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>ΔS&lt;sup&gt;0&lt;/sup&gt; (J mol&lt;sup&gt;-1&lt;/sup&gt;K&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>5.29 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.81 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.94</td>
<td>-24.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>302</td>
<td>6.52 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4.20 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.96</td>
<td>-26.73</td>
<td>48.0</td>
<td>2.49 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>309</td>
<td>8.74 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>7.95 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.99</td>
<td>-28.99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.
Binding constants of CBB-BSA system before and after the addition of site probes.

<table>
<thead>
<tr>
<th>K without the site probe (M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K with warfarin (M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K with ibuprofen (M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K with digitoxin (M&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.20 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>7.73 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.22 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4.19 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>