Indian Journal of Chemistry
Vol. 55A, July 2016, pp. 820-823

Investigations on interaction between atazanvir sulphate and bovine serum albumin by fluorescence spectroscopy

Umesh S Mote[a,] * & G B Kolekar[b]

[a]PG Department of Physical Chemistry, Karmaveer Bhaurao Patil College, Urun-Islampur, Tal. Walwa, Dist. Sangli 415 409, Maharashtra, India

Email: moteumesh@gmail.com

[b] Department of Chemistry, Shivaji University, Kolhapur 416 004 Maharashtra, India

Received 29 October 2015; revised and accepted 30 June 2016

The interaction between bovine serum albumin (BSA) and atazanvir sulphate (AS) has been investigated under the physiological *p*H 7.4 condition using fluoresence spectroscopy. The binding constant, number of binding site, thermodynamic parameters such as ΔG, ΔH, ΔS and nature of binding forces between BSA-AS have been obtained from the steady state fluorescence quenching of BSA by AS. The static quenching is confirmed from Stern-Volmer quenching constant at different temperatures. The effect of AS on the conformation of BSA has been analyzed using synchronous and three-dimensional fluorescence spectroscopy.

Keywords: Fluorescence quenching, Thermodynamic parameters, Synchronous fluorescence, Binding constant

Bovine serum albumin (BSA), a large globular protein (66,000 Da), consists of a single chain of 583 amino acids residues, and forms sub-domains by paired 17 disulfide bonds. It is formed from three domains I, II and III, divided into two sub-domains (A and B), and contains two tryptophan residues, Trp-134 and Trp-212, located respectively in domains I and II. Trp-212 residue is surrounded by a hydrophobic environment within a protein pocket while Trp-134 residue is located in a hydrophilic environment, close to the protein surface[1]. Serum albumin, as one of the most abundant carrier proteins, plays an important role in the transport and disposition of endogenous and exogenous compounds present in blood. Distribution and metabolism of many biologically active compounds such as metabolites, drugs and other organic compounds in the body are correlated with their affinities towards serum albumin, and the binding ability of drugs-albumin in blood stream may have a significant impact on free concentration and metabolism of drugs[2,3]. Strong binding can decrease the concentration of free drug in plasma, whereas weak binding can lead to a short lifetime or poor distribution. Consequently, the investigation of the binding between drugs and serum albumin is of fundamental importance in pharmacology and pharmacodynamics. Therefore, the binding of drugs to serum albumin *in vitro*, considered as a model in protein chemistry to study the binding behavior of proteins, has been an interesting research field in chemistry, life sciences and clinical medicine[4-7].

Atazanavir sulfate (AS) is a white to pale-yellow crystalline powder. It is slightly soluble in water (4-5 mg/mL, free base equivalent) and the *p*H of its saturated solution in water is about 1.9 at 24±3 °C. Atazanavir, a protease inhibitor (PI), is approved in many countries for use as a component of antiretroviral therapy (ART) regimens for the treatment of adult, and in some countries in paediatric patients with HIV-1 infection[8].

In the present study we have made a detailed and insightful study on interaction between BSA and AS using the combination of steady state fluorescence, synchronous fluorescence and three-dimensional fluorescence. The mechanism of binding of drug to protein mainly focussing on binding sites available on protein for drug molecule and nature of interaction is discussed.

Experimental

Fluorescence emission and excitation spectra were recorded on a PC based spectrofluorophotometer (Shimadzu RF-5301 PC) equipped with a xenon lamp source and 1.0 cm quartz cell. Synchronous and three-dimensional fluorescence spectra were recorded on FP-8300 spectrofluorometer (JASCO Japan) equipped with 1.0 cm quartz cell. The sample masses were accurately weighted using a microbalance (Denver Instruments with 0.1 mg accuracy).

Atazanvir sulphte was obtained as a gift sample from Lupin Pharma (Pune, India). BSA was purchased from Himedia Chemicals. The stalk solution of atazanvir sulphate (1×10^{-4} mol^{-1} L^{-1}) was prepared by dissolving it first in a small amount of methanol and then diluted to appropriate volume with tris-HCl

buffer solution of *p*H 7.4. BSA solution (1×10^{-5} mol^{-1} L^{-1}) was prepared by dissolving in tris-HCl buffer solution of *p*H 7.4. All the chemicals were of analytical grade and doubly distilled water was used throughout.

The drug-protein solutions were prepared by mixing appropriate quantities of AS and BSA solution. The BSA concentration was fixed at 1×10^{-6} mol L^{-1}, while the AS concentration was varied from 0 to 8.18×10^{-5} mol L^{-1}. Fluorescence quenching spectra were recorded at 300 K in the range of 285-450 nm at excitation wavelength of 280 nm. Both the excitation and emission bandwidth were kept constant at 5 nm. The experiments were repeated to study fluorescence quenching at 310 K and 320 K.

Results and discussion

BSA can emit strong intrinsic fluorescence at 339 nm on excitation at 280 nm, and the sole Trp and Tyr residues in BSA explains this phenomenon. The intrinsic fluorescence of BSA is very sensitive to its microenvironment and when the local surroundings of BSA was altered slightly, its intrinsic fluorescence would weaken obviously. Factors such as protein conformational transition, biomolecule binding, and denaturation, etc., are responsible for the observed weakening.

Figure 1 shows the fluorescence quenching of BSA induced by AS. It is observed that the fluorescence intensity of BSA decreased regularly with increasing concentration of AS, with the maximum fluorescence emission wavelength undergoing an obvious blue shift from 339 nm to 335 nm. This may be due to a change in the conformation of BSA as a result of the interaction of BSA with AS. This viewpoint was further confirmed by synchronous and 3D fluorescence spectra discussed below. The quenching of fluorescence may be static or dynamic and can be recognized by the temperature dependence studies.

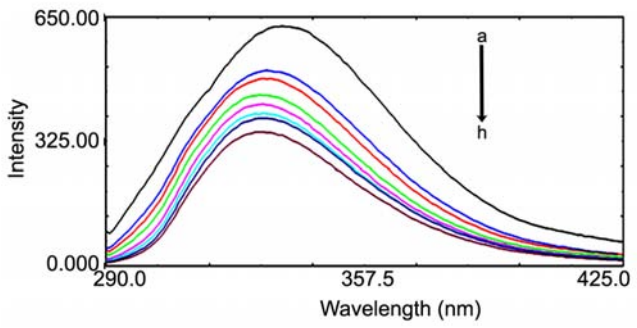

Fig. 1 – Effect of AS on fluorescence spectrum of BSA. {[BSA] = 1×10^{-6} mol L^{-1}; [AS] = 0 – 8.18×10^{-5} mol L^{-1} (a to h)}.

The quenching rate constants are expected to decrease with increase in temperature for static quenching while for dynamic quenching reversed effect was observed[9]. The fluorescence quenching data were analyzed by Stern-Volmer equation, $F_0/F = 1 + K_{sv}[Q]$, where F_0 and F are the fluorescence intensity in the absence and presence of quencher, K_{sv} and [Q] are Stern-Volmer quenching constant, and concentration of quencher respectively. The Stern-Volmer plots at various temperatures show a good linear relationship (Fig. 2). As it is known, linear Stern-Volmer plots represent a single quenching mechanism, either static (a formation of a complex between quencher and fluorophore) or dynamic (a collisional process)[10]. Linear fittings of the experimental data to the SV equation afforded K_{SV} and K_q. K_{SV} increases from 0.893 at 300 K to 1.381 at 320 K. This indicates that the fluorescence quenching of BSA is likely to occur via a dynamic quenching mechanism. However the values of quenching rate constant (K_q) which were evaluated using the equation, $K_q = K_{SV}/\tau_0$, where τ_0 is the average lifetime of the protein without the quencher. The value of τ_0 of the biopolymer[11] is 10^{-8} s^{-1} and hence the values of K_q were of the order of 10^{12} L mol^{-1} s^{-1}. It is two orders of magnitude greater than the maximum diffusion collision quenching rate constant (2.0×10^{10} L mol^{-1} s^{-1}) for a variety of quenchers with biopolymer[12]. Therefore, this suggests that the fluorescence quenching process of BSA may be mainly governed by a static quenching mechanism arising from a complex formation[13] rather than a dynamic quenching mechanism.

When small molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant (K) and the numbers of binding sites (n) can be determined by the following equation[14]:

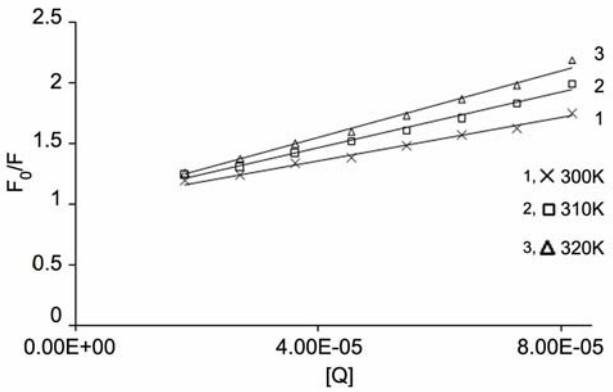

Fig. 2 – Stern-Volmer plots describing BSA quenching by AS at different temperatures. [1, 300; 2, 310; 3, 320].

$$\log\frac{F_0 - F}{F} = \log K + n\log[Q] \quad \ldots (1)$$

The plots of $\log\frac{F_0 - F}{F}$ versus log [Q] for the BSA-AS system at different temperature are shown in Fig. 3. The plots exhibit good linearity and the values of number of binding sites and binding constant for BSA- AS system are presented in Table 1. The results show that the binding constants increased with increase in temperature, indicating that the binding is an endothermic reaction[15] and BSA-AS complex is formed with rising temperature. The values of n are approximately equals to unity, indicating the existence of just a single binding site in BSA for AS.

The thermodynamic parameters dependent on temperatures were analyzed in order to study the binding forces between AS and BSA. Generally, four types of forces are involved in the binding of small molecule with macromolecules, viz., of hydrogen bonds, van der Waals forces, electrostatic forces and hydrophobic interaction forces. If the temperature does not vary significantly, the enthalpy change (ΔH) can be regarded as a constant. The free energy change (ΔG) can be estimated from the following equations, based on the binding constants at different temperature[16],

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad \ldots (2)$$

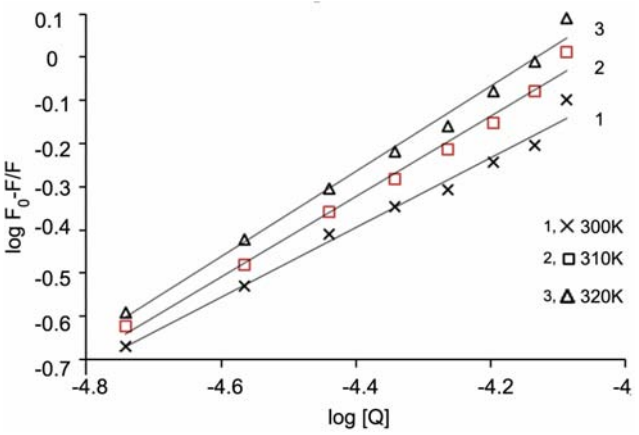

Fig. 3 – Plots of log (F_0-F/F) versus log[Q] at different temperatures. [1, 300; 2, 310; 3, 320].

$$\Delta G = \Delta H - T\Delta S \quad \ldots (3)$$

where K is the binding constant, R is the gas constant ΔS is the entropy change and T is the experimental temperature. The van't Hoff plot is linear and the enthalpy change and entropy change were obtained from slope and intercept respectively as 23.21 kJ mol^{-1} and 94.35 J mol^{-1} K^{-1}. The negative values of free energy change (−28.28, −29.22 and 30.17 kJ mol^{-1} at 300, 310 and 320 K respectively) indicates that the interaction between BSA and AS takes place spontaneously. Positive ΔH and ΔS value indicate the less dominant hydrogen bond formation and predominant hydrophobic force between AS and BSA[17] respectively. Therefore, hydrophobic interactions are assumed to play a major role in the binding of AS to BSA.

The conformational changes of BSA was evaluated by synchronous fluorescence intensity of protein amino acid residues before and after the addition of AS. Synchronous fluorescence spectroscopy can give information about the molecular environment in the vicinity of the fluorophore molecules in low concentration under physiological condition. When the D-value ($\Delta\lambda$) between excitation and emission wavelength are stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine or tryptophan residues respectively[18]. It is observed from the Fig. 4 that when $\Delta\lambda$ is fixed at 60 nm, the emission spectrum shows the

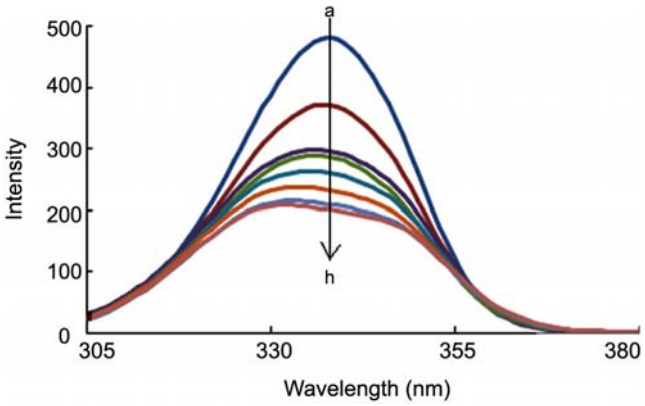

Fig. 4 – Effect of the AS on synchronous fluorescence spectra of BSA. {$\Delta\lambda$ = 60 nm; [AS] = 0.0 to 8.18×10^{-5} mol L^{-1} (a to h)}.

Table 1 – Stern-Volmer quenching constants, quenching rate constants, binding constant and binding sites for the interaction between AS and BSA at various temperatures

T (K)	K_{SV} (×10^4 L mol^{-1})	K_q (×10^{12} L mol^{-1} s^{-1})	R	K (x10^4 L mol^{-1})	n	R
300	0.8934	0.8934	0.988	0.1479	0.8134	0.986
310	1.1578	1.1578	0.990	0.5847	0.9290	0.990
320	1.3810	1.3810	0.991	1.1967	0.9870	0.989

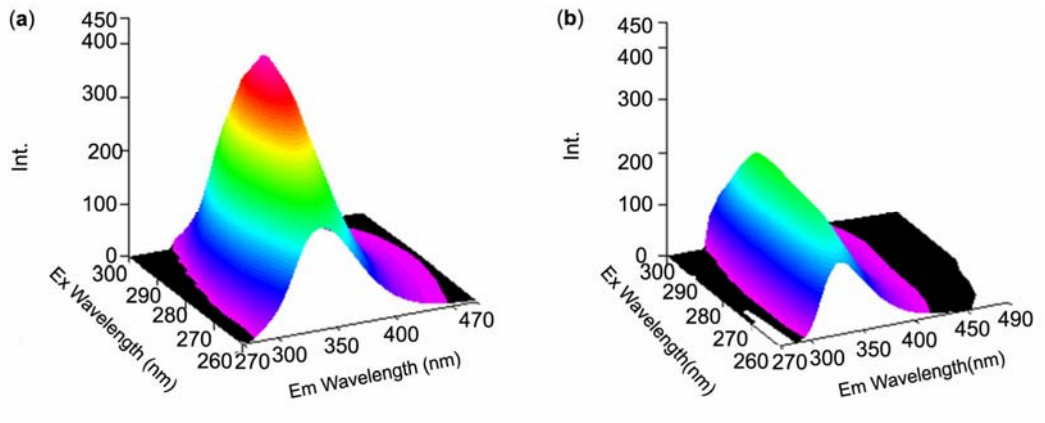

Fig. 5 – Three-dimensional fluorescence spectra on addition of AS to BSA. [(a) BSA = $1x10^{-6}$ mol L^{-1}; (b) BSA-AS complex: BSA = 1×10^{-6} mol L^{-1}; AS = 8.18×10^{-5} mol L^{-1}].

significant blue shift from 338 nm to 330 nm and intensity is quenched with the continuous addition of AS. The blue shift effect indicates that the polarity around tryptophan residue is decreased and the hydrophobicity is increased and the micro-environment around the tyrosine residue remains unaltered during the binding of AS to BSA.

Additional evidence regarding the conformational changes of BSA in the presence of AS was obtained from the three-dimensional fluorescence (TDF) spectra. The conventional TDF spectrum was usually expressed as the change of fluorescence intensity with both the excitation and emission wavelength. TDF spectrum has superiority in the study of fluorescent probes because the molecular information of fluorescent substance can be estimated by the finger printing characterstics, the peak positions and fluorescence intensity of TDF spectra[19]. The fluorscence intensity decreases drastically in the presence of AS in the present study (Fig. 5). The results show that the TDF map of BSA and BSA-AS are different obviously. From the decrease of fluorescence intensity of the peaks in combination with the synchronous fluorescence spectra results, we can conclude that the interaction of AS with BSA induces a slight unfolding of the polypeptides of the protein, which results in a conformational change of the protein that increase the exposure of some hydrophobic regions that were previously buried.

The present study shows that hydrophobic interactions play key roles in the process of the binding of AS with BSA, which indicates that the drug can efficiently interact with BSA. From the synchronous fluorescence spectra and three dimensional spectra it was found that the conformation of BSA undergoes a change upon interaction with AS. These results indicate that the drug can penetrate into the hydrophobic cavity in the subdomain IIA of BSA where Trp212 is located and that tryptophan group (Trp212) in the subdomain IIA is the binding site of AS on the serum albumin.

Acknowledgement

USM gratefully acknowledges the financial support (No. F. 47-635/13WRO) from University Grants Commission (WRO) Pune, India.

References

1 Peters Jr T, *Adv Protein Chem,* 37 (1985) 161.
2 Carter D C & Ho J X , *Adv Protein Chem,* 45 (1994) 153.
3 Zsila F, Bikadi Z & Simonyi M, *Biochem Pharmacol*, 65 (2003) 447.
4 Chatterjee S & Srivastava T S, *J Porphyrin Phthalocyanin,* 4 (2000) 147.
5 Hu Y J, Liu Y, Wang J B, Xiao X H & Qu S S, *J Pharm Biomed Anal,* 36 (2004) 915.
6 Hu Y J, Liu Y, Shen X S, Fang X Y & Qu S S, *J Mol Struct,* 738 (2005) 143.
7 Wang Y Q, Zhang H M, Zhang G C, Tao W H, Fei Z H & Liu Z T, *J Pharm Biomed Anal,* 43 (2007) 1869.
8 Croom K F, Dhillon S & Keam S J, *Drug,* 69 (2009) 1107.
9 Lackowicz J R, *Principles of Fluorescence spectroscopy*, 3rd Edn, (Plenum Press, New York) 2006, pp. 277-350.
10 Eftink M R & Ghiron C A, *J Phys Chem,* 80 (1976) 486.
11 Lakowicz J R & Weber G, *Biochemistry,* 12 (1973) 4161.
12 Ware W R, *J Phys Chem,* 66 (1962) 445.
13 Jin J & Zhang X, *J Lumin,* 128 (2008) 81.
14 Mote U S, Bhattar S L, Patil S R & Kolekar G B, *Luminescence,* 25 (2010) 1.
15 Wang Y-Q, Zhang H-M, Zhang G-C, Tao W-H & Tang S-H, *J Mol Struct,* 830 (2007) 40.
16 Mote U S, Han S-H, Patil S R & Kolekar G B, *J Lumin,* 130 (2010) 2059.
17 Ross P D & Subramanian S, *Biochemistry,* 20 (1981) 3096.
18 Miller J N, *Proc Anal Div Chem Soc,* 16 (1979) 203.
19 Yan Y, Xu J & Chen G, *Sci China,* 39 (1996) 527.