Chapter – 3: Fluorescence studies on drug interaction with model globular proteins

3.1. Background

Protein-ligand interactions are important in the distribution and transport of small drug molecules in living systems. Therefore, understanding the molecular basis of these interactions is indispensable towards designing of new and more efficient specific therapeutic agents for improved drug action [1,2]. Fluorescence spectroscopy is one of the most widely used techniques to achieve this goal because of its high sensitivity coupled with easy operational procedure. The modular structural domain of HSA to bind a series of exogenous drugs led to an intense research towards understanding the forces responsible as well as comparative thermodynamic parameters for drug binding etc. Also, a common trend is to note the drug binding domain by comparative experimental studies with site specific markers [3, 4] in conjunction with molecular docking results [5-7]

Quenching measurement of intrinsic protein fluorescence is an important method to investigate the interactions of drugs with proteins. It can reveal the accessibility of quenchers to albumin’s fluorophore groups, mainly tryptophan (Trp) and/or tyrosin (Tyr); and helps to understand the binding mechanism of albumins with drugs and provide clues to the essential of binding phenomenon. In this work, BSA, HSA and LYS are selected as the model proteins because of their medicinal importance, low cost, commercial availability, and unusual ligand-binding properties [8-10]. BSA is 66 kDa protein containing two tryptophan residues that possess intrinsic fluorescence, Trp212 is located within a hydrophobic binding pocket of the protein and Trp134 is located on the surface of the molecule [11-13]. The structural homologue HSA is a protein with the molecular weight of 66,500 containing 585 amino acid residues. However, there is only a single Trp residue within HSA at the position of 214 in domain II, which makes it very convenient to study the protein intrinsic fluorescence [14-16]. On the other hand, lysozyme is an antimicrobial protein widely distributed in various biological fluids and tissues including avian egg and animal secretions, human milk, tears, saliva, airway secretions, and secreted by polymorphonuclear leukocytes [17]. It has many physiological and pharmaceutical functions, such as anti-inflammatory, anti-viral, immune modulatory, anti-histaminic and anti-tumor
activities [18-22]. So it is extensively used in the pharmaceutical and food fields. Intriguingly, LYS also has the capacity to carry drugs and it can cure some illness via the binding with these drugs. It is a 14.6 kDa single chain protein and is formed by 129 amino acid residues, including α-helix, β-sheet, turns and disorder. It contains six Trp residues, four disulfide bonds and three tyrosine molecules in its structure [23-25]. Three of the Trp residues are located at the substrate binding sites, two in the hydrophobic matrix box, while one is separated from the others [26]. Among the amino acid residues Trp62 and Trp108 are the most dominant fluorophores, both being located at the substrate binding sites [27].

In view of the multifarious functions of BSA, HSA as well as LYS and their important practical role from a medicinal point of view, studies on the interactions between drugs and these proteins have important meaning on realizing the transport and metabolism process of the drugs, the relationship of structure and function of proteins, and the chemical essence of the interaction between biological macromolecule with small molecule.

3.2. Sulfadiazine – protein interaction studied by fluorescence spectroscopy and molecular modeling

3.2.1. Introduction

Sulfadiazine (4-amino-N-pyrimidin-2-yl-benzenesulfonamide, SDZ) is a short-acting antibacterial drug of the parent sulfonamide class of synthetic antibiotic compounds. It eliminates bacteria that cause infections by stopping the production of folate inside the bacterial cell, and is commonly used to treat urinary tract infections (UTIs) [28]. Also SDZ, in combination with an anti-malarial drug pyrimethamine, is frequently used to treat toxoplasmosis in warm-blooded animals like immuno-compromised HIV-positive individual patients [29]. The therapeutic use of sulfonamide antibiotics is restricted in veterinary field because of the health risk associated with consumption of sulfonamide residue contaminated animal products [30, 31]. Furthermore, hypersensitive patients who suffer a reaction after taking a sulfonamide drug are thought to be at increased allergic risk for other drugs as well [32], although the mechanism of sulfonamide-related reactions is poorly understood till date. Also, exposure to sulfonamide residues to the consumers for prolonged period is known to cause the development of drug-resistant bacteria [33]. Keeping these in mind, in this section we report the results of our investigation on the mechanism of interaction of SDZ with model water soluble proteins like bovine as well as human
serum albumins (BSA and HSA, respectively) and lysozyme (LYS) by fluorescence quenching method.

**Figure 3.1:** Variation of Trp fluorescence intensity of BSA with increasing concentration of SDZ. [SDZ]/μM = 0(i), 22(ii), 44(iii), 66(iv), 88(v), 110(vi), 154 (vii), 198 (viii), 242 (ix). Concentration of protein was kept fixed at 12 μM.

### 3.2.2. Quenching of Trp fluorescence in presence of Sulfadiazine.

Intrinsic Trp fluorescence of BSA, HSA as well as LYS appears at 340, 350 and 338 nm, respectively. In all the cases, the fluorescence intensity is quenched regularly with the addition of SDZ. Fig. 3.1 shows a representative example of the fluorescence emission spectra of BSA titrated with different concentrations of SDZ in pH 7.4 buffer solutions. Comparison of the normalized fluorescence curves (data not shown) show no apparent difference in spectral position and width, even on highest concentration of SDZ addition in the respective cases while comparing with the protein fluorescence in absence of any SDZ. In the inset of Fig. 3.1, the variation of F₀/F with SDZ concentration is also shown. Interestingly, the SV plot is linear till [SDZ] = 100 μM and shows deviation from linearity and moves upward beyond this concentration in all the proteins. The linear fitting of the quenching data in the lower concentration regime is shown.
by the solid line in the inset of Fig. 3.1. Interestingly, the fluorescence quenching behavior and
the deviation from linearity remains identical for all the proteins within the experimental
temperature range of 298-323 K. The magnitude of SV quenching constant (K_{SV}) obtained from
the slope of this linear plot is listed in Table 3.1 for all the proteins. While the value of K_{SV} is
found to be almost constant over the whole temperature range for albumins (BSA & HSA), there
is a slight decrease in the corresponding value for LYS with increase in temperature (K_{SV}/10^3, M^{-1}
= 17.6 and 12.1 at 298 and 323 K, respectively). The decrease in K_{SV} at higher temperature
indicates that the additional quenching mechanism responsible for the upward curvature (see
below) in the SV plot is significant at higher concentration of SDZ for LYS in comparison with BSA
and/or HSA. Before we discuss more about the origin of the upward curvature in SV plot, it is
worth noting the effect of SDZ addition on the intrinsic Trp fluorescence lifetime of the three
proteins.

### Table 3.1. Quenching constants (K_{SV}) for the linear part of the Stern-Volmer plot at different
temperatures for BSA, HSA and LYS

<table>
<thead>
<tr>
<th>Temp. (K)</th>
<th>BSA</th>
<th>HSA</th>
<th>LYS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_{SV}</td>
<td>S.D.</td>
<td>R</td>
</tr>
<tr>
<td>298</td>
<td>8.76±0.41</td>
<td>0.03</td>
<td>0.99</td>
</tr>
<tr>
<td>303</td>
<td>9.18±0.37</td>
<td>0.03</td>
<td>0.99</td>
</tr>
<tr>
<td>308</td>
<td>8.87±0.37</td>
<td>0.03</td>
<td>0.98</td>
</tr>
<tr>
<td>313</td>
<td>8.91±0.45</td>
<td>0.04</td>
<td>0.99</td>
</tr>
<tr>
<td>318</td>
<td>8.86±0.45</td>
<td>0.04</td>
<td>0.98</td>
</tr>
<tr>
<td>323</td>
<td>9.00±0.41</td>
<td>0.03</td>
<td>0.98</td>
</tr>
</tbody>
</table>

*K_{SV} values are represented in the unit of 10^3 M^{-1}, the error limit indicates the confidence
interval in three independent measurements; Standard deviation (S.D.) and correlation
coefficient (R) is also given in each case.

#### 3.2.3. Analysis of Trp fluorescence lifetime in presence of Sulfadiazine.

Fig. 3.2 shows the time-resolved data of Trp fluorescence decay for HSA and LYS
obtained by exciting the sample at 295 nm and monitoring the fluorescence at the respective
emission maximum. The corresponding data for BSA is almost identical to that of HSA. It is seen
that in all the three cases, the experimental data needs more than one exponential fit to give
acceptable statistical parameters. The decay times and the corresponding amplitude
contributions for the two exponential fitting in BSA and HSA are 2.53 ns (74%), 6.55 ns (26%) and
3.43 ns (71%), 6.67 ns (29%), respectively. On the other hand for LYS, minimum three
exponential decay function was necessary for adequate fitting of the experimental decay data. The corresponding fitting parameters are 0.77 ns (67%), 2.11 ns (31%) and 4.5 ns (3%). The nature of the fluorescence decay and also the experimentally obtained parameters are consistent with the literature reported values [34-37]. Nevertheless, the measured decay represents the radiative relaxation process of Trp fluorophore(s) for these systems and difference in the fluorescence decay time indicates the difference in location of the Trp residue(s) in the protein structure. The origin of the multiple fluorescence decay times, even for proteins containing single Trp residue like HSA, has attracted considerable attention since almost four decades ago [38,39] and till continues to be a topic of intense research activities [40]. It is now more or less accepted that the multi-exponential Trp fluorescence decay is mainly the outcome of fluorophore heterogeneity resulting from the formation of several microstates and is related to either a rotamer [41, 42] or an exciplex model [43].

Figure 3.2: Time-resolved fluorescence decay traces (open circle) of LYS in buffer solution of pH 7.4 along with the fitting data (solid line) and instrument response function (IRF) ($\lambda_{\text{exc}} = 295$ nm). Distribution of weighted residual and autocorrelation function as well as numerical values of reduced chi-square ($\chi^2$) and Durbin-Watson (DW) parameter is also shown for different fitting functions.

We have calculated the $\tau_{\text{av}}$ from the measured fluorescence decay data in all the three proteins with different concentrations of SDZ. It was found that $\tau_{\text{av}}$ practically remains constant
(τ₂ = 4.53, 4.82 and 1.9 ns for BSA, HSA and LYS, respectively) over the whole range of quencher concentration. The detail tabulation of the fluorescence decay data in each quencher concentration for all three proteins is given in Table 3.2. The non-variance of fluorescence decay data confirms the static quenching mechanism in all these cases. Therefore, the slope obtained from the linear fitting of the low quencher concentration data (inset of Fig. 3.1) is representative of binding constant (Kₛ).

**Table 3.2.** Fluorescence decay time (τ) of BSA, HSA and LYS titrated with different concentrations of SDZ. [SDZ] /μM = 0, 22, 44, 66, 88, 110, 154, 198 and 242 from top to the bottom row. The excitation wavelength is 295 nm and the emission monitored at the respective fluorescence maxima.

<table>
<thead>
<tr>
<th></th>
<th>BSA</th>
<th></th>
<th></th>
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</tr>
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<td>a₂</td>
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<tr>
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<td>6.7</td>
<td>27</td>
<td>4.8</td>
<td>0.8</td>
<td>2.1</td>
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<td>6.5</td>
<td>27</td>
<td>4.4</td>
<td>3.6</td>
<td>6.6</td>
<td>28</td>
<td>4.9</td>
<td>0.7</td>
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<td>0.8</td>
<td>2.1</td>
</tr>
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<td>27</td>
<td>4.5</td>
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<td>4.6</td>
<td>3.8</td>
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<td>21</td>
<td>4.9</td>
<td>0.9</td>
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<td>3.9</td>
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<td>4.0</td>
<td>7.5</td>
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<td>4.8</td>
<td>1.2</td>
<td>2.6</td>
</tr>
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<td>12</td>
<td>4.8</td>
<td>0.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* The decay times (τ/ns) are within the error limit ±0.1 ns; the values in parenthesis are the corresponding amplitudes (in %); τₐv indicates the average lifetime (ns) calculated using Eq. 2.33.

### 3.2.4. Upward curvature in S-V plots.

The data points obtained in fluorescence quenching experiments are nicely fitted with the simulated curve using Eq. 2.17 as shown for a representative case of LYS in Fig. 3.3. The calculated value for the radius of the quenching sphere is ca. 10.4 and 10.9 Å for HSA and BSA, respectively at 298 K. However, for LYS, the corresponding parameter is found to be approximately 12.0 Å. The values are somewhat greater than the sum of the estimated radii of the Trp (fluorophore) and SDZ (quencher) molecule. However, it is already reported in the literature that the quenching partners not necessarily to be in close contact for the quenching sphere model to operate [44]. This is due to the fact that once the quencher is inside the sphere it can diffuse through the distance separating the fluorophore and the quencher during excitation and still show the transient effect. Interestingly, the magnitude of Rₛ increases about 10% with change in temperature from 298 K to 318 K. This observation is consistent if it is
hypothesized that with increase in temperature, the quencher can cover even a longer distance to show the transient effect.

**Figure 3.3.** SV plots for the interaction of Sulfadiazine with LYS. The solid line represents the simulation of the experimental points using quenching sphere model.

3.2.5. Binding constants and number of binding sites.

From the slope and intercept of the simulated linear plot using Eq. 2.36, the binding constant, K and number of binding sites, n can be obtained for different protein/SDZ combination at various temperatures. The corresponding values are displayed in table 3.3.
Table 3.3. Association constant (K) and number of binding sites (n) obtained from the double-log plot of interaction of SDZ with BSA, HSA and LYS at different temperature.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Temp (K)</th>
<th>BSA</th>
<th></th>
<th></th>
<th>HSA</th>
<th></th>
<th></th>
<th>LYS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
<td>n</td>
<td>S.D.</td>
<td>K</td>
<td>n</td>
<td>S.D.</td>
<td>K</td>
<td>n</td>
<td>S.D.</td>
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<tr>
<td>298</td>
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<td>0.02</td>
<td>3.57</td>
<td>1.2</td>
<td>0.03</td>
<td>20.12</td>
<td>1.3</td>
<td>0.12</td>
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<tr>
<td>303</td>
<td>4.99</td>
<td>1.1</td>
<td>0.03</td>
<td>4.06</td>
<td>1.1</td>
<td>0.03</td>
<td>29.84</td>
<td>1.3</td>
<td>0.12</td>
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<tr>
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<td>0.04</td>
<td>47.03</td>
<td>1.4</td>
<td>0.07</td>
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<td>1.2</td>
<td>0.03</td>
<td>4.19</td>
<td>1.1</td>
<td>0.05</td>
<td>45.49</td>
<td>1.4</td>
<td>0.05</td>
</tr>
<tr>
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<td>1.2</td>
<td>0.02</td>
<td>4.46</td>
<td>1.1</td>
<td>0.04</td>
<td>64.41</td>
<td>1.4</td>
<td>0.04</td>
</tr>
<tr>
<td>323</td>
<td>14.65</td>
<td>1.2</td>
<td>0.03</td>
<td>5.00</td>
<td>1.1</td>
<td>0.04</td>
<td>89.71</td>
<td>1.5</td>
<td>0.07</td>
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</table>

\textsuperscript{a}K values are represented in the unit of $10^4$ M$^{-1}$; S.D. denotes Standard deviation; correlation coefficient (R) is 0.99±0.01 in each case.

The binding constant value (K /$10^4$ M$^{-1}$) for HSA, BSA and LYS with SDZ are found to be approximately 3.6, 4.2 and 20.1, respectively at 298 K. The magnitude of n in case of albumins varies within the range 1.15±0.05. Efficient quenching of Trp fluorescence in presence of the drug clearly indicates a strong interaction between Trp of all the proteins and SDZ. In the albumins, Trp-214 is located in sub-domain IIA, which is a well-characterized binding cavity for small organic molecules. Also, it indicates that SDZ interact with Trp-214 only. In case of BSA, although it contains two tryptophan molecules, the other tryptophan residue (Trp-134, located in domain I) remains inaccessible to the water soluble ligands. On the other hand, relatively higher value of n (1.40±0.10) in case LYS indicates mutual interaction from both the Trp residues (Trp62 and Trp108) in the ligand binding site with SDZ. It is interesting to note the variation of K value with increase in temperature for the three different proteins table 3.3. While the magnitude of K in HSA increases only by a factor of ca. 1.4 by increasing the temperature from 298 K to 323 K, the corresponding increase is about 3.5 and 4.5 times in the cases of BSA and LYS, respectively. As discussed above, both BSA and LYS contains multiple Trp residues in the binding cavity in contrast to a single Trp residue for HSA. The difference in the increase in K values in the former cases can be regarded as due to some positive cooperative effect in the binding process at elevated temperature. Similar observation was also reported recently [45], where it was shown that the chemiluminescent probe luminol binds cooperatively only in the ligand binding domain of BSA but not in HSA.
3.2.6. Thermodynamics of protein-SDZ interaction: Nature of forces responsible for the association.

The interaction forces between the drugs and bio-molecules may include electrostatic interactions, formation of multiple hydrogen bonds, van der Waals interaction, hydrophobic and steric contacts within the antibody binding site etc. [46]. The sign and magnitude of the thermodynamic parameters can account for the main factor(s) responsible towards the stability of drug – protein complex [47]. The enthalpy (ΔH) and entropy (ΔS) change was calculated from the slope and intercept of the van’t Hoff plot at six different temperatures, within 298 to 323 K range; whereas, Gibb’s free energy change (ΔG) can be estimated from Eq. 2.37. The representative plots are shown in Fig. 3.4 and all the thermodynamic parameters are collected in table 3.4.

![van’t Hoff plot for the binding of SDZ with BSA, HSA and LYS](image)

**Figure 3.4: van’t Hoff plot for the binding of SDZ with BSA, HSA and LYS**

The negative value of the free energy change (ΔG) is indicative of a spontaneous binding of these ligands to the proteins. Further, this exothermic process is accompanied by positive ΔS values in all the cases. A positive ΔS value is often indicative of a hydrophobic mechanism in drug – protein interaction [47]. Specific electrostatic interaction among ionic species in solution is characterized by positive ΔS and negative ΔH values; whereas, negative entropy and enthalpy
changes indicate the importance of van der Waals force as well as hydrogen bond formation. From the results displayed in table 3.4, it is clear that the hydrophobic interaction plays the major role in SDZ binding in all the cases. Interestingly, the net entropy change in the case of BSA and LYS is almost twice than that in HSA. This may be due to the fact that the binding in HSA is mostly in the single binding site (domain IIA) and the resulting interaction is due to the presence of only one Trp in the protein. Recent report on the interaction of sodium sulfadiazine with HSA also predicts the involvement of sub-domain IIA as the principal binding region [48]. However, in case of both the BSA and LYS, the interaction is stronger due to the presence and involvement of more than one Trp sites with the drug molecule. The present argument is also manifested in lower values of the binding constant (K), number of binding sites (n) and free energy change (ΔG) in HSA table 3.3 & 3.4 in comparison with BSA and LYS. Nevertheless, the whole binding process is considered as entropy driven in all the cases and the increase in entropy due to the ligand binding might be due to the destruction of the protein secondary structure.

**Table 3.4.** Relative thermodynamic parameters for the interaction of SDZ with BSA, HSA and LYS.

<table>
<thead>
<tr>
<th>Temp</th>
<th>BSA</th>
<th>HSA</th>
<th>LYS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔG</td>
<td>ΔH</td>
<td>ΔG</td>
</tr>
<tr>
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<td>-26.0±0.2</td>
<td>48.4±4.1</td>
<td>-26.0±0.3</td>
</tr>
<tr>
<td>303</td>
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<td>ΔS=</td>
<td>-26.6±0.2</td>
</tr>
<tr>
<td>308</td>
<td>-28.5±0.1</td>
<td>0.25±0.01</td>
<td>-27.2±0.1</td>
</tr>
<tr>
<td>313</td>
<td>-29.8±0.1</td>
<td>-27.8±0.2</td>
<td>-34.2±0.1</td>
</tr>
<tr>
<td>318</td>
<td>-31.0±0.1</td>
<td>-28.4±0.1</td>
<td>-35.5±0.1</td>
</tr>
<tr>
<td>323</td>
<td>-32.2±0.2</td>
<td>-29.1±0.2</td>
<td>-36.8±0.2</td>
</tr>
</tbody>
</table>

* Gibb’s free energy (ΔG) and enthalpy (ΔH) changes are given in kJ mol⁻¹ and entropy (ΔS) change is given in kJ mol⁻¹ K⁻¹.

3.2.7. Molecular docking studies.

The best energy ranked results of SDZ binding with BSA and LYS are shown in Fig. 3.5. The binding result for HSA and the orientation of the drug (SDZ) is quite similar to that in case of BSA. The modeling results suggested that SDZ interacts with both the albumins (BSA & HSA) at site I in subdomain IIA, and the interaction between them was mainly dominated by a hydrophobic force, which was in agreement with the binding mode proposed in thermodynamic
analysis. Furthermore, the docking results show that the location of SDZ is about 10.8 Å from the Trp214 in HSA (for BSA, it is ca. 10.1 Å from Trp213); whereas, the distance ranges within 4.1 to 6.7 Å from the three Trp residues in the binding site of LYS. These results provide a good structural basis to explain the fluorescence quenching of the proteins in presence of SDZ and in nice agreement with the calculated distance parameters from the Stern-Volmer plots, particularly for albumins. However for LYS, the difference between the experimental and calculation data indicates a more complex nature of interaction between the fluorophore and SDZ, possibly due to the presence of three tryptophan moieties in close vicinity at the binding site. Nevertheless, the free energy change of binding (ΔG) obtained from the docking simulations was -29.9, -30.5 and -27.6 kJ mol⁻¹, for BSA, HSA and LYS respectively which are in good agreement with the experimentally obtained values. The little difference between these two results may be due to exclusion of solvent in docking simulations and/or rigidity of the receptor other than tryptophan.

Figure 3.5: Minimum energy docking structure of SDZ with BSA (left) and LYS (right). The zoomed representation of the orientation of the drug (SDZ) and the distance (Å) from the tryptophan (Trp) residues in the binding site is also given in each case.

3.2.8. Conclusion

The interaction between model water soluble proteins like bovine and human serum albumins (BSA and HSA, respectively) and lysozyme (LYS) with sulfadiazine (SDZ) were studied by monitoring the intrinsic tryptophan fluorescence quenching of proteins in presence of the ligand at different temperatures. The results show that the binding pattern of SDZ is similar in all the proteins indicating a preferential interaction at the hydrophobic sites of protein residue. The binding interaction is relatively strong with an apparent association constant ca. 10⁴ M⁻¹ and
responsible for the efficient Trp fluorescence quenching. Estimation of thermodynamic parameters indicates that the spontaneous complexation occurs through an entropy driven pathway. The structural basis of fluorescence quenching and the calculated thermodynamic parameters were corroborated with molecular docking calculation results. The knowledge of interaction can lead to better understanding of the mechanism of sulfonamide related reaction in warm blooded animals and also binding as well as transport properties of these model proteins toward the widely used anti-bacterial drug like SDZ at the molecular level.

3.3. Caffeine and sulfadiazine interact differently with human serum albumin

3.3.1. Introduction

Human serum albumin (HSA) is the most widely distributed protein in human blood plasma with a negatively charged surface. It binds to a series of chemically diverse exogenous and endogenous compounds with moderate to high affinity ($10^4$ to $10^6$ M$^{-1}$) and also acts as transport capacity enhancer of blood [49-51]. Caffeine (1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione, CAF) is a bitter, white crystalline xanthene. Caffeine is the world’s most widely consumed psychoactive substance, particularly in the Western countries, and believed to stimulate central nervous system (CNS). In fact, caffeine and its derivatives are known antagonists for both $A_1$ and $A_2$ – adenosine receptors in brain preparations; although with varying degree of effectiveness [52]. Caffeine influences the body water excretion mechanism when consumed in sufficient doses. It is evident that the adverse effect of caffeine ingestion (with LD$_{50}$ = 127 mg/kg) is manifested only in the event of at least 20-30 times overdose to reach the concentration level of ca. > 50 mg/L (ca. 250 µM) than those found in normal diet [53, 54], although some reports observe that caffeine concentration in human circulatory plasma rarely exceeds ca. 100 µM [55]. The peak plasma caffeine level in humans reach normally within 15-45 min after ingestion [56]; however, serum caffeine reaches to the level as low as <0.8 mg/L (ca. 5 µM) only after 24 hr. of abstention [57]. Furthermore, results suggest that caffeine may be associated with enhanced reactions to some psycho-stimulants like nicotine [58] even in moderate dose. The cytotoxic and/or cytostatic effects of the DNA-IA cancer drugs doxorubicin and novantrone, in different cell lines are reported to decrease substantially in presence of CAF [59, 60]. Being the principal component of coffee, CAF
is also found to be partly responsible for increased homocysteine concentration in human plasma with potential risk for cardiovascular diseases [61].

In view of the multifarious functions as well as medicinal importance, studies on the interactions of SDZ as well as CAF drugs with HSA protein seem to be very important. In fact, binding studies of both these drugs with proteins were reported using different techniques [62-67]. However, while some of the earlier experiment [63] for the interaction of CAF with HSA was lacking proper correction of inner filter effect in the fluorescence intensity data [62]; the relatively recent result published by Zhang et. al. [65] limits the CAF concentration range only upto 45 µM, which is far below than the physiological concentration range of CAF consumers (ca. 100 - 500 µM). Similarly, Ali et. al. [67] reported the interaction of SDZ with HSA with a maximum concentration range of about 175 µM; almost half of the free physiological concentration of the drug as mentioned before. Furthermore, in all the reports mentioned above, the quenching of intrinsic protein fluorescence was ambiguously assigned to be of static type without any time-resolved measurement.

In this section, we report the interaction of SDZ and CAF with HSA by monitoring intrinsic protein fluorescence quenching method by steady state and time-resolved fluorescence measurements. The thermodynamic parameters, nature of forces responsible for drug binding and location of the probe in macromolecular architecture were discussed based on the results of temperature variation experiments as well as molecular docking results. In spite of binding in the same structural cleft (Sudlow’s site 1) of HSA; the fluorescence quenching mechanism, the extent and also the nature of forces responsible for drug binding, orientation of the drugs relative to Trp214 in sub-domain IIA are all distinctly different for these two drugs.

3.3.2. Trp fluorescence quenching in presence of drugs

The tryptophan (Trp) fluorescence intensity of HSA decreases continuously with regular addition of both SDZ and CAF without any significant change in the spectral profile. This indicates that there is no conformation change in the HSA secondary structure which accounts for similar environment (polarity) around the tryptophan residue in presence of various concentrations of CAF and/or SDZ. SV equation was applied to analyze the change in emission intensity data at various concentrations of quenchers at different temperatures. The respective plots show different behavior with increasing quencher concentration. While for CAF the SV plot remains linear throughout, a positive deviation from linearity is noted for SDZ beyond the quencher concentration of about 150 µM. Positive deviations from the Stern-Volmer equation
are frequently observed when the extent of quenching is large. In that case, the Stern-Volmer plot exhibits an upward curvature, concave toward the y-axis at higher [Q]. These positive deviations may be an indication of two distinct situations: firstly, the fluorophore can be quenched by both mechanisms with the same quencher; and secondly, the upward curvature can also indicate the presence of a sphere of action. The later assumes the existence of a sphere of certain volume around the fluorophore within which a quencher will cause quenching with a probability of unity. In this situation, quenching occurs due to the quencher being adjacent to the fluorophore at the moment of excitation. These closely spaced fluorophore-quencher pairs are immediately quenched, but fluorophores and quenchers do not actually form a ground-state complex. This type of apparent static quenching is usually interpreted in terms of the model "sphere of action". Before we discuss more about the difference in quenching mechanism in the present cases, it is worth noting the effect of the drugs on the fluorescence lifetime of HSA.

3.3.3. Analysis of Trp fluorescence lifetime with addition of SDZ and CAF

The intrinsic Trp fluorescence decay of HSA was monitored in presence of different concentrations of both the quenchers. In all the cases, the experimental data needs more than one exponential fit to give acceptable statistical parameters as discussed in the previous section. The nature of the multi-exponential fluorescence decay of HSA, even in absence of any quencher, is consistent with the literature reports [37-39] and is mainly related to either a rotamer [41, 68] or an exciplex model [43]. It is to be noted here that the lifetime of the individual components increase slightly (about 0.6 – 0.8 ns) in presence of both SDZ and CAF, which seems little unusual and inconsistent with the observation that the static fluorescence intensity of HSA quenches in presence of these drugs. It is already well-known that several factors may contribute on the variation of Trp fluorescence lifetime of HSA [69-71]. These include (i) conformational heterogeneity of the protein structure, (ii) extent of interaction of the fluorophore and water molecules, and (iii) interaction of the Trp moiety with nearby amino-acid residues. It is rather difficult to isolate any particular point specifically for the reason of increase in individual lifetime component; however, it is more likely that the change in local water structure in presence of added drugs is responsible for this. Contributions from the other two can be ignored because of the non-variance of the steady state spectrum with addition of these drugs as mentioned before. So, the slight increase in lifetime components is attributed to the increase in fractions of water-exposed Trp residues generated locally without any significant change in the protein structure. The calculated values of average lifetime ($\tau_{av}$) with different
concentrations of SDZ and CAF are found to be practically constant over the whole range of quencher concentration. The non-variance of HSA fluorescence decay time confirms the static quenching mechanism in both these cases.

3.3.4. Modeling the difference in fluorescence quenching

The fluorescence quenching data shows linear variation of SV plot in case of CAF at different temperatures. However, as mentioned earlier, positive deviation in SV plot suggests the involvement of additional quenching process at higher concentration of SDZ. Interestingly, in a recent publication, Ali et al. [67] reported the quenching of HSA fluorescence by SDZ to follow simple linear SV relationship. This is due to the fact that the maximum concentration of SDZ used in their study was ca. 150 µM and the positive deviation in SV plot appears only beyond this concentration. The additional contribution towards extra fluorescence quenching leading to positive deviation in SV plot may be related to the presence of a quenching sphere. The fitting of
experimental data points with the simulated curve using this model results the radius of the quenching sphere as approximately 10.4 Å at 298 K.

**Table 3.5.** Relative thermodynamic parameters for the interaction of CAF and SDZ with HSA.\(^a\)

<table>
<thead>
<tr>
<th>Temp</th>
<th>(K) /10^2</th>
<th>(\Delta G)</th>
<th>(\Delta H)</th>
<th>(\Delta S)</th>
</tr>
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<td></td>
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<td>9.59</td>
<td>-17.6±0.1</td>
<td></td>
<td></td>
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<td>9.29</td>
<td>-17.9±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>323</td>
<td>9.37</td>
<td>-18.2±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>328</td>
<td>9.14</td>
<td>-18.4±0.2</td>
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<table>
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<th>(\Delta G)</th>
<th>(\Delta H)</th>
<th>(\Delta S)</th>
</tr>
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<td>0.12±0.01</td>
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<tr>
<td>328</td>
<td>5.00</td>
<td>-29.1±0.2</td>
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</tbody>
</table>

\(^a\) Gibb’s free energy (\(\Delta G\)) and enthalpy (\(\Delta H\)) changes are given in kJ mol\(^{-1}\) and entropy (\(\Delta S\)) change is given in kJ mol\(^{-1}\) K\(^{-1}\).

**Figure 3.7:** Determination of binding constant (\(K\)) and number of binding sites (\(n\)) at various temperatures for HSA-SDZ system.
3.3.5. Thermodynamics of protein-drug interaction: Nature of forces responsible for the association

The combination of several weak forces play a vital role in describing the association of organic molecules into the drug binding site of the protein which include electrostatic interaction, hydrogen bond formation, van der Waals interaction, hydrophobic and steric contacts etc. [46]. Screening of the main force(s) responsible in describing the stability of a particular drug-protein complex can be obtained by following the method described in the seminal report of Ross and Subramanian [47].

The results of fluorescence quenching experiments with addition of both CAF and SDZ at different temperatures are analyzed based on SV equation. For CAF, the $K_s$ values obtained from the slope of the linear SV plot and displayed in Fig. 3.6 can be considered as the pure ground state association constant (K) and are listed in table 3.5. However for SDZ, the $I_0/I$ values show upward curvature at higher quencher concentration in all the temperatures studied here. The linear variation (double log plot) of SDZ binding with $n = 1.15 \pm 0.05$ at different temperatures are shown in Fig. 3.7. The binding constant values calculated from the intercept of these straight lines are also given in table 3.5. It is to be noted that although the 1:1 binding stoichiometry for these drugs with HSA reported in this study match closely with the recent literature [65, 67], the binding constant values differ almost by an order of magnitude. However, as mentioned before, the maximum concentrations of the drug used in the previous studies, i.e. ca. 45 µM for CAF [65] and ca. 175 µM for SDZ [67], was almost half of the normal physiological concentration range for adult humans. Therefore, it can be assumed that the drug binding constant and also the corresponding thermodynamic parameters calculated in those studies do not corroborate with standard physiological condition necessary for understanding the drug-protein interaction. Furthermore, this observed difference may also result from a different albumin concentration used for fluorescence titration with the drugs. Whereas, the working concentration of the protein was not specifically mentioned in SDZ-HSA system [67], it was as low as approximately 3 µM for CAF-HSA system reported by Zhang et. al. [65]. In contrast, we have used 12 µM protein concentration in the present investigation. It is well known that the binding affinity of the serum albumin depends strongly on the albumin concentration as well as buffer composition of the medium [9]. For example, Weisiger et. al. reported almost six fold decrease of HSA binding affinity towards bilirubin on increasing the protein concentration from 18 to 320 µM [72].
The corresponding van't Hoff plots for HSA binding with CAF and SDZ are shown in Fig. 3.8 and the calculated thermodynamic parameters are given in table 3.5. The negative value of the free energy change (ΔG) indicates a spontaneous binding process of both these drugs to the protein. It is well-known that a positive ΔS value corresponds to a hydrophobic mechanism in drug – protein interaction [47]. On the other hand, electrostatic interaction among ionic components is characterized by positive ΔS along with negative ΔH values. The results given in table 3.5 indicate that the calculated binding constant values are about two orders of magnitude higher for SDZ in comparison with CAF. Furthermore, the hydrophobic interaction plays the major role in SDZ binding with HSA; however for CAF, the electrostatic interaction seems to be predominant force for binding.

![Figure 3.8: van't Hoff plot for the binding of SDZ and CAF with HSA.](image)

Almost two orders of magnitude higher binding constant of HSA with SDZ in comparison with CAF can be rationalized on the basis of the difference in their physicochemical properties. It is well known that acidic/anionic drugs bind more strongly with albumin and the extent of binding strongly correlates with the lipophilicity of the drugs [73, 74]. The ionic state of the drug (percentage of fraction ionized) in a working buffer solution of known pH can be estimated from the pKₐ values of the drug using the modified form of Henderson – Hasselbach equation [75].
\[
\% \text{ of ionised fraction} = \frac{10^{-pK_a}}{10^{-pK_a} + 10^{-pK_a'}} \times 100
\] (3.1)

On the other hand, a qualitative estimate of lipophilicity can be obtained from the standard P or logP values of the drugs; where, P represents the ratio of the distribution of the drug in organic phase (usually octanol) to that in aqueous phase [76]. Noting the pK_a and logP values for SDZ (6.36, 0.25 to 0.39) and CAF (10.4, -0.24 to -0.55), respectively [77], it is believed that SDZ is expected to interact strongly with HSA at the physiological pH = 7.4, which is consistent with the observation made in the present study.

It is to be noted here that alike SDZ, the CAF also binds with 1:1 stoichiometric ratio. This may be due to the fact that the binding in HSA is mostly in the single binding site (domain IIA). The single tryptophan (Trp214) molecule in HSA, which essentially acts as the fluorescent label in this study, is also present in the same domain. This results a strong interaction of the drug with the fluorophore and results an efficient quenching of Trp fluorescence. However, the orientation of the drug (see below for molecular docking results) in the binding site and the corresponding forces responsible for drug binding seem to be different in the two cases.

3.3.6. Molecular docking results

The best energy ranked results of CAF and SDZ binding with HSA are shown in Fig. 3.9. The modeling results suggested that both these drugs interact with the protein at site I of sub-domain IIA. However, a close look at the binding region and the orientation of the drugs reveal the difference. While SDZ is mainly surrounded by Arg209, Ala213, Gly328, Lys351, Ala350 residues in one side of the Trp214; the location of CAF is just in opposite side of the binding domain and encompasses mainly the residues Ala291, Ser287, Arg257, and Glu292 etc. in its vicinity. Considering CAF to be a strongly electron deficient system in conjunction with the known anti-oxidant behavior of HSA (capable of donating electrons) [9, 78], the complex formation in the protein binding site through electrostatic interaction seems feasible. However, in SDZ-HSA case, the hydrophobic interaction plays the major role as indeed predicted by the fluorescence experiments. These results explain the intrinsic fluorescence quenching of HSA in presence of CAF as well as SDZ from structural view-point of the drug-protein complex and shed light on the significant difference in the binding behavior of these two drugs with HSA. The free energy change of binding (ΔG) obtained in the lowest energy conformation from the docking simulations was -30.5 and -27.1 kJ mol⁻¹, for SDZ and CAF respectively. These results differ quantitatively from the experimentally obtained values; however, the qualitative trend of the
binding affinity of these two drugs towards HSA is nicely corroborated with the experimental observation. The little difference between the fluorescence and molecular modeling results may be due to exclusion of solvent in docking simulations or rigidity of the receptors other than tryptophan.

![Image: Minimum energy docking structure of HSA with SDZ and CAF. Inset shows the zoomed representation of the orientation of the drugs relative to Trp214 residue in the binding site (sub-domain IIA) of the protein.]

3.3.7. Conclusions

The interaction of sulfadiazine (SDZ) and caffeine (CAF) with human serum albumins (HSA) was studied by monitoring the intrinsic tryptophan fluorescence quenching of protein in presence of the drugs at different temperatures. The results confirm that although both these drugs bind preferentially to sub-domain IIA in the binding domain of the protein, the magnitude of interaction and the forces responsible for binding differ significantly. The hydrophobic binding in SDZ-HSA system is almost two orders of magnitude stronger than the feeble electrostatic interaction in CAF-HSA system. The extent of binding observed in fluorescence experiments correlates nicely with the ionizing tendency ($pK_a$) and lipophilicity ($\log P$) of the drugs. The structural basis of intrinsic fluorescence quenching and the calculated thermodynamic
parameters were corroborated with molecular docking calculation results. The knowledge can lead to better understanding towards the mechanism of the binding processes as well as transport properties of these two drugs in human plasma at the molecular level.

3.4. References