

Isothermal Titration Calorimetry and Molecular Dynamics Simulation Studies on the Binding of Indometacin with Human Serum Albumin

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ABSTRACT

Human serum albumin (HSA) is the most abundant protein in the blood plasma. Drug binding to HSA is crucial to study the absorption, distribution, metabolism, efficiency and bioavailability of drug molecules. In this study, isothermal titration calorimetry and molecular dynamics simulation of HSA and its complex with indometacin (IM) were performed to investigate thermodynamics parameters and the structural changes induced by the ligand binding, respectively. To estimate the binding affinity of drug molecule to subdomains IB and IIA in HSA protein, binding free energies were calculated using the Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA). The binding association constant (K) and the standard Gibbs free energy changes (ΔG°) of indometacin binding to the protein obtained from ITC technique are $9.12 \times 10^3 \text{ M}^{-1}$ and $-22.59 \text{ kJ mol}^{-1}$, respectively. All results indicate that the binding affinity of the drug molecule to subdomain IIA is more than that of subdomain IB of HSA. Thus the obtained thermodynamics characteristics, hydrophobic forces most likely played a major role, but hydrogen bonding also could not be ignored. One of the most important finding is that the subdomain IIA of HSA is the main binding site for indomethacin that confirmed by molecular dynamics simulation.

Keywords: Human serum albumin (HSA), Isothermal titration calorimetry (ITC), Molecular dynamics simulation (MD), Indomethacin (IM), Molecular mechanics poisson-boltzmann surface area (MM-PBSA)

INTRODUCTION

Human Serum Albumin (HSA) is the most abundant protein in blood plasma plays a key role in the transport of metabolites, fatty acids [Fanali *et al.*, 2012], ions [Bal *et al.*, 2013], vitamins [Memarpoor-Yazdi and Mahaki, 2013], steroids and foreign molecules such as drugs [Fasano *et al.*, 2005]. The knowledge of interaction mechanism between plasma proteins and drugs is of crucial importance for understanding the pharmacokinetics and pharmacodynamics of a drug [Bertucci and Domenici, 2002; Yang *et al.*, 2007; Sleep *et al.*, and Yamasaki *et al.*, 2013; Yang *et al.*, 2014; Dangkoob *et al.*, 2015]. The primary structure of HSA is well-known and its first high-resolution crystal structure was obtained by He *et al.* [He and Carter, 1992]. HSA is comprised of 585 amino acids and has a molecular mass of 66.5 kDa [Meloun *et al.*, 1975].

HSA has an asymmetric heart-shaped molecule [He and Carter, 1992]. The tertiary structure of HSA is composed of three homologous domains: domain I (residues 1-195), domain II (residues 196-383), and domain III (residues 384-585). Each domain is divided into two subdomains (A, B) that contain six and four α -helices, respectively [Carter and Ho, 1994; Sugio *et al.*, 1999]. The main drug binding sites on HSA protein are situated in subdomains IIA and IIIA, namely site I [Ascenzi *et al.*, 2005] and site II [Isogai and Hiray, 2013], respectively. Site I is dominated by strong hydrophobic interaction with most bulky, neutral heterocyclic compounds, while site II binds mainly by van der Waals and/or hydrogen-bonding interactions with many aromatic carboxylic acids. The multiple binding sites in HSA imply to the special ability of HSA to interact with many organic and inorganic molecules.

Non-steroidal anti-inflammatory drugs, NSAIDs are a class of drugs that provides analgesic (pain-reducing) and antipyretic (fever-reducing) effects, and in higher doses,

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anti-inflammatory effects [Clara *et al.*, 2014]. These drugs are transported using HSA protein. NSAIDs are non-narcotic and thus are used as a non-addictive alternative to narcotics. Indometacin (IM) a methylated indole derivative with chemical formula ($C_6H_{16}ClNO_4$) is a power non-steroidal anti-inflammatory drugs (NSAIDs) which Individuals treated with such drugs are relieved of fever, pain, stiffness and swelling [Wojnarowska *et al.*, 2009]. In recent years, it has been shown to have anti-tumor activity. Clinical epidemiological in- visitations have demonstrated a markedly reduced incidence and mortality for colon cancer among patients with regular IM consumption [Gridley *et al.*, 1993]. Also, there are some reports that indomethacin is useful in a treatment of closing of the open artery in premature infants [Sekar and Corff, 2008] and in treatment of jaundice disease in infants [Shankaran *et al.*, 1982 and Lam *et al.*, 1990]. It is believed that IM to act by inhibiting cyclooxygenase enzymes (COX-I and COX-II) suppressing prostaglandin synthesis, relieving pain and reducing temperature in febric patients 10-14, 2012 [Campanile *et al.*, 1996; Kalgutkar *et al.*, 2000]. There are many investigations in this field using Chromatography [Salary and Hadjmohammadi, 2015], fluorescence spectrophotometry [Zhang *et al.*, 2012, Tayyeb *et al.*, 2015 and Mohseni-Shahri *et al.*, 2016], differential scanning calorimetry [Faroongsang, 2016] and molecular modeling [Tayyeb *et al.*, 2015] methods. These experimental studies have revealed that the hydrophobicity is the most important physicochemical property in the interaction between HSA and most of the drugs [Xu *et al.*, 2005]. The general conclusion obtained from these studies shows that the formation of the HSA-drug complex has induced major protein conformational changes with a reduction of α -helix and an increase of β -structure. The major reduction of the α -helix is characteristic of a partial protein unfolding in these HSA-drug complexes [Tajmir-Riahi *et al.*, 2007].

Isothermal titration calorimetry (ITC), directly measures energetics associated with the binding reaction and yields accurate information on various thermodynamic parameters associated with the binding process, as binding constant, stoichiometry, enthalpy, and entropy from a single experiment [Dam and Brewer, 2002]. On the other hand, many scientists have used recently the molecular dynamics simulation (MD) method to investigate the molecular basis

of proteins as well as protein-ligand interactions [Artali *et al.*, 2005; Li *et al.*, 2010; Sudhamalla *et al.*, 2010; Castellanos and Colina, 2013]. Application of MD methods give insight on the atomic details and at the appropriate time scale that are concealed from the most experimental methods such as X-ray crystallography, neutron diffraction and nuclear magnetic resonance (NMR). In addition, MD simulations have been used as a complementary tool for the experimental techniques. The importance of HSA in biological systems and indomethacin in medicine motivated us to investigate the effect of the binding of indometacin on structure of HSA and the stability of a protein-drug complex system in aqueous solution. Understanding molecular recognition requires knowledge of the thermodynamic properties of the system, together with insight into the structure and dynamics of the complex of interest. Here, ITC experiments and MD simulations have been used to study the binding of indomethacin with HSA protein.

MATERIAL AND METHODS

Materials

Human serum albumin (HSA, 99%, fatty acid free) and indometacin, purchased from Sigma Alderich, was used without further purification. HSA was dissolved in phosphate buffer solution (100 mM, pH 7.4). The stock solution of drug was prepared by dissolving it in the phosphate buffer to form 16.8 mM solution and the solution of HSA was prepared at the same buffer to have a concentration of 0.21 mM. All other chemical materials were of analytical grade.

Apparatus and Methods

Isothermal titration calorimetry (ITC). ITC experiments were performed using VP-ITC Microcalorimeter (MicroCal, LLC, Northampton, MA, USA) at 25 °C. This device has two cells with volume of 1.8 ml and a sample injection syringe with volume of 300 ml. 1.8 ml of HSA (0.21 mM) was loaded into the sample cell. The reference cell contained 100 mM Tris buffer (pH 7.4). The protein in the sample cell was titrated with indometacin (16.8 mM) diluted in alcohol-buffer mix in the syringe (alcohol content in sample cell did not exceeded 3% v/v in the last injection). The titration of the protein with

drug involved 30 titers, the first injection volume of 5 μl and 29 next injection volumes of 10 μl . The heat flow is a measure of the interaction. In each experiment, the heat effect of injection an alcohol buffer mixture into the protein was obtained in the same temperature to subtract from the heat of protein-drug titration. MicroCal Origin software was used to fit the ITC data and find binding parameters [Sultan and Swamy, 2005; Wiseman *et al.*, 1989].

Molecular dynamics simulation (MD). The starting structure was obtained from the Protein Data Bank [Berman *et al.*, 2000], PDB code 1AO6 to HSA simulation [Sugio *et al.*, 1999] and PDB code 2BXM to HSA-Indometacin simulation [Ghuman *et al.*, 2005], which is in complex with the myristate and indomethacin (IMN) and water molecules. All myristate and water molecules were discarded. In this PDB file, there are two indometacin molecules, one at IB subdomain and another at IIA subdomain. Submitted molecules are initially optimised by Guassian software at the HF/STO-3G or AM1 [Dewar *et al.*, 1985] or PM3 [Stewart, 1989]) level of theory, then re-optimised at the B3LYP/6-31G* level of theory [Becke, 1993; Lee *et al.*, 1988; Perdew and Wang, 2013] to assign the charges in the topology file. The charges are then further refined (within the uncertainty in the charge assignment) to obtain neutral charge groups. All simulations were carried out using the Gromacs 4.6.5 of program [Berendsen *et al.*, 1995; Van der spoel *et al.*, 2005] with the gromos53a6 force field [Oostenbrink *et al.*, 2004]. Recently, a web accessible Automated force field Topology Builder (ATB; <http://compbio.biosci.uq.edu.au/atb/>) and Repository [Malde *et al.*, 2011] has been developed to provide interaction parameters for a wide range of molecules compatible with the GROMOS force field [Oostenbrink *et al.*, 2004]. Each initial structure was then placed into a rectangular periodic box of SPC [Berendsen *et al.*, 1981] water molecules such that the distance between the closest atom of the solute and the edges of the water box was at least 10 Å in all directions. Then, 20 sodium ions were added to maintain the electroneutrality of the systems. Periodic boundary conditions were used in all three directions. Long-range electrostatics interactions were evaluated with the particle mesh Ewald (PME) method [Batcho *et al.*, 2001]. The cutoff distance for non-bonded interactions was set to 10 Å. Initial velocities were assigned

from a Maxwell-Boltzmann distribution at the reference temperature. All bonds involving hydrogen atoms were constrained using the SHAKE algorithm [Ryckaert *et al.*, 1977]. The time step for all MD simulations was set 2 fs. In all of the simulations, the temperature and pressure were controlled by the V-rescale temperature coupling scheme [Bussi *et al.*, 2007] and the Parrinello-Rahman protocol [Parrinello and Rahman, 1981], respectively.

The following protocol was used for all MD simulations: First, 10000 steps steepest descent minimization was performed. After minimization, system was heated from 0 to 298 K through a canonical ensemble (NVT)-MD simulation for 1000 ps. To adjust the solvent density, a short simulation (1000 ps) was carried out in the NPT ensemble. Finally, a production run was performed for 30 ns under conditions of constant pressure and temperature. In equilibration and production run steps, target temperature and pressure are 298 K and 1 bar, respectively. Molecular graphics were made with the VMD program [Humphrey *et al.*, 1996].

RESULTS AND DISCUSSION

ITC Analysis of Indometacin Binding to HSA

ITC is a straightforward method to determine detailed thermodynamic descriptions and better investigate the mechanism of the binding interaction between the heavy metal and protein. We can get information about standard enthalpy changes (ΔH°), standard entropy changes (ΔS°), binding stoichiometry (n), and binding affinity constant (K) by ITC technique. ITC results recorded during titration of HSA solution with IMN solution were shown in Fig. 1 after correction for dilution effects, in which experimental titration curves analyzed with the single set of binding site model. The derived thermodynamic parameters were presented in Table 1 Based on Fig. 1. The number of binding site (n) is equal to 7.8 (near to 8) and binding constant is equal to $9.12 \times 10^3 \text{ M}^{-1}$.

According to Table 1, a negative value of ΔH° ($-9.72 \text{ kJ mol}^{-1}$) suggested the interaction between indometacin and HSA was an exothermic process. The negative values of ΔG° ($-22.59 \text{ kJ mol}^{-1}$) indicated that the binding interaction was a spontaneous reaction with approximately 8 thermodynamical binding sites at 298 K. On the other hand,

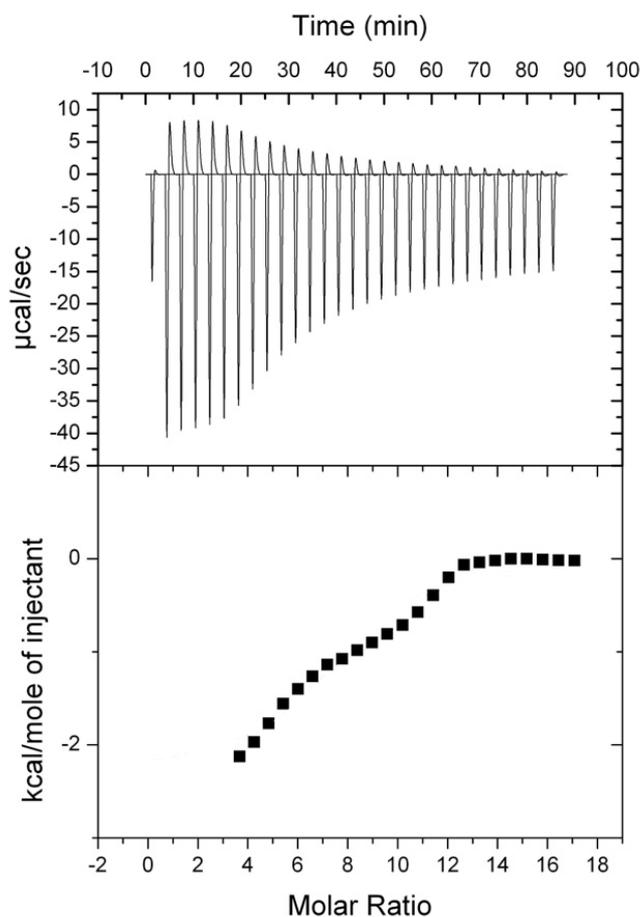


Fig. 1. ITC profiles of the interaction between indometacin [21 mM] and HSA [0.21 mM] at 298 K. The top panel showed the original data of sequential titration of indometacin solution into HSA solution in Tris buffer (100 mM, pH = 7.4). The bottom panel presented integrated heat results of the titration after correction for dilution heats against the molar ratio of indometacin-HSA.

Table 1. The Binding Parameters Obtained from ITC Method for IM-HSA Interaction at 298 K

N	K	ΔH°	ΔS°	ΔG°
(Site mol^{-1})	(M^{-1})	(kJ mol^{-1})	($\text{J mol}^{-1} \text{K}^{-1}$)	(kJ mol^{-1})
7.8 (~8)	9.12×10^3	-9.72	43.19	-22.59

the positive value of ΔS° ($43.19 \text{ J mol}^{-1} \text{K}^{-1}$) revealed that hydrophobic interaction was the predominant driven force in this reaction [Subramanian, 1981]. Additionally, the binding affinity constant value suggested that the binding of

indometacin and HSA was a relatively moderate process, compared with strong ligand-protein interactions with K values ranging from 10^7 - 10^8 [Charbonneau and Tajmir-Riahi, 2010]. The value of the number of binding sites is

nearly 8 site mol⁻¹ that confirmed by previous study using differential scanning calorimetry [Zini *et al.*, 1979]. From the perspective of pharmacokinetics, the moderate affinity of indometacin for HSA induces a faster diffusion rate to reach its target site in the reaction system [Feroz *et al.*, 2012; Li and Yang, 2015].

Generally, the interaction forces between small organic molecules and biological macromolecules can include the hydrophobic force, hydrogen bonds, the van der Waals force, electrostatic interactions, *etc.* [Leckband, 2000]. Ross and Subramanian [Ross and Subramanian, 1981] have characterized the signs and magnitudes of the thermodynamic parameters associated with various kinds of interactions that may take place in the protein association process. It was observed that formation of the HAS-IM complex was a favorable process with a negative value of ΔG° . The negative value of ΔH° observed in this experiment cannot be attributed mainly to electrostatic interactions, since the value of ΔH° is very small. A negative ΔH° value is frequently taken as evidence for hydrogen bond formation in the binding interaction, and the hydrogen bond can be formed from the structure of IM, while a positive value of ΔS° is frequently taken as evidence for a hydrophobic interaction. For the HAS-IM system, it was found that the main contribution to ΔG° arises from ΔS° rather than from ΔH° . Thus, from the thermodynamics characteristics summarized above, hydrophobic force most likely played a major role, but hydrogen bonding also could not be excluded [Zhang *et al.*, 2009; Rahman *et al.*, 1993].

MD Simulation of Indometacin Binding to HSA

In the first part of this study, to investigate structural changes induced by the ligand (IMN) binding, molecular dynamics simulations of free HSA (585 residues) and HSA-IMN complex for both of drug molecules (IMN1 and IMN2) were performed and then compared. In this study, root mean square deviations (RMSD), radius of gyration (Rg), solvent accessible surface area (SASA), H-bond numbers and secondary structure (DSSP) were investigated as a measure of the structural properties. Using these properties, the stability of the HSA protein can be qualitatively compared during the simulation time.

Root mean square deviations (RMSD). The time evolution of RMSD from the initial structure was calculated

for three production run simulations (HSA, HSA-IMN1 and HSA-IMN2 complexes). RMSDs of the protein C α atoms are plotted in Fig. 2 showing RMSD for the free protein and protein in complex with IM molecules. The RMSD value of the HSA protein fluctuated around 0.37 nm in free protein and 0.52 nm and 0.41 nm in IMN1 and IMN2, respectively. As shown in Fig. 2, RMSD values for protein C α atoms are increased upon binding. Ligand binding cause the increase of RMSD value of protein in complex with respect to free protein. As it's seen in Fig. 2, all these simulations as the same other biological systems have the same logical manner in their fluctuations (rmsd: 0.3-0.6 nm) and probably the structure of the protein was more destabilized in the binding to IMN1 (0.52 nm) compared with IMN2 (0.41 nm).

Radius of Gyration (Rg) and solvent accessible surface area (SASA). The radius of gyration is an indicator of the compactness of a protein [Lobanov *et al.*, 2008; Galzistkaya *et al.*, 2008; Tsai and Nussinov, 1997]. The proteins that have less radius of gyration, will access the less molecular surface area to the exposed solvent and the exposure of the protein to water is decreased when its structure becoming more compact [Zhao *et al.*, 2006 Zunliang *et al.*, 2014].

The average values of radius of gyration and the hydrophobic, hydrophilic and total accessible surface areas of the protein are listed in Table 3. As it is seen in Table 3, otherwise all values are similar, but in MD simulations we can not ignore even barely changes in the findings. The value of Rg is somewhat increased in the binding to IMN1 (2.74 nm) and somewhat decreased in the combination to IMN2 (2.72 nm). As it is expected, by reducing the radius of gyration and increasing the compactness rate in the binding to IMN2, the less area of the protein can be exposed to the solvent (323 nm²). All these findings align with the previous studies [Zhao *et al.*, 2006 Zunliang *et al.*, 2014]. By making a comparison between the changes of hydrophobic and hydrophilic surface values in Table 3, it becomes obvious that otherwise all of them have the same trend, but the hydrophobic interaction changes has most important physicochemical property in this interaction.

Hydrogen bond numbers. One of the most important analysis is hydrogen-bond numbers to evaluate the stability of a protein. The analysis of hydrogen-bond numbers in Table 3 indicates that, in the binding to IMN2, an increase in the number of hydrogen bonds in this protein was

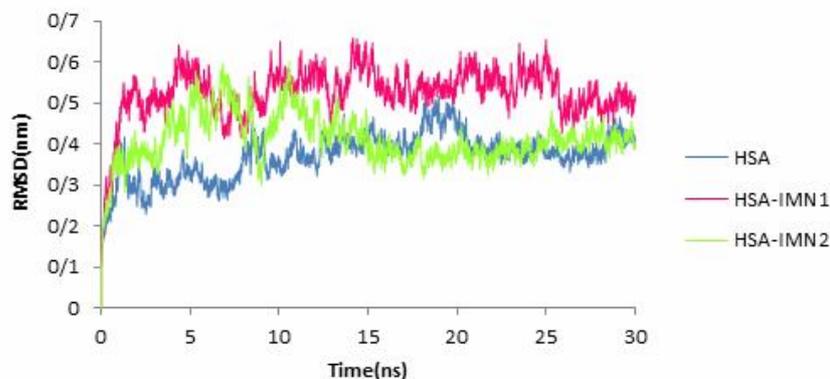


Fig. 2. Root mean square deviation (RMSD) measured from the corresponding starting structure in HSA structure (blue color) and HSA in complexes with IM1 (red color) and IM2 (green color).

Table 2. The Mean of Radius of Gyration (Rg), the Hydrophobic and Hydrophilic and Total Solvent Accessible Surface (SASA) and H-Bond Numbers in Free HSA and HAS Complexes with IM Molecules

	HSA	HAS-IMN1 (Subdomain IB)	HAS-IMN2 (Subdomain IIA)
RMSD (nm)	0.37	0.52↑	0.41↑
Rg (nm)	2.73	2.74↑	2.72↓
Hydrophobic surface area (nm ²)	186	194	189
Hydrophilic surface area (nm ²)	139	140	134
Total surface area (nm ²)	325	335↑	323↓
H-Bond numbers	453	444↓	454↑

happened (454 vs. 453). It's seen *vice versa* effect for IMN1(444 vs. 453).

This finding in agreement to other results indicate that the more stability of HSA protein in the binding to indomethacin in subdomain IIA (site I) compared subdomain IB.

Dictionary of the secondary structure of the protein (DSSP). The analysis of the secondary structure was done by DSSP program [Kabsch W, Sander, 1983]. The secondary structures of free protein and protein in complex with IMN ligands (IMN1 and IMN2 complexes) as a

function of time are depicted in Fig. 3. To distinguish between the secondary structures types, different colors were used. The overall secondary structure pattern of free and bound HSA is maintained during the 30 ns MD simulation, although there was slight change at some points as a function of time. With a glance to Fig. 3, the first molecule of drug (IMN1) binding somewhat decreased α -helix content of HSA and the second molecule (IMN2) somewhat increased α -helix content which is in agreement with other results. Also, the binding of indomethacin to HSA deduce to the bend structure appearing in the both of

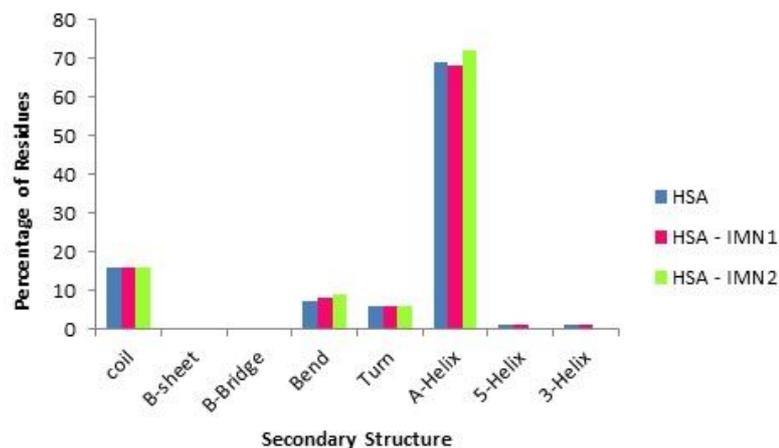


Fig. 3. Secondary structures as function of time for 30 ns simulation, at 298 K for HSA (blue color), HSA-IMN1 (red color) and HSA-IMN2 (green color) complexes.

Table 3. The Binding Free Energies and their Components for HSA-Indometacin Complexes at T = 298 K

Energy (KJ mol ⁻¹)	Van der walls energy	Electrostatic energy	Polar solvation energy	SASA energy	Binding energy
HAS+IMN1 (subdomain IB)	-158.288	-1426.732	577.17	-16.743	-1024.58
HAS+IMN2 (subdomain IIA)	-68.847	-1908.667	826.025	-9.820	-1161.31

complexes in compared with free protein.

Binding free energy. To estimate the binding affinity of IMN molecules to HSA protein, binding free energies were calculated using MM-PBSA method to complement the structural analysis. To obtain the mean values of binding free energies with reasonable precision [51,52], a total of all snapshots were collected from the MD simulations of the HSA-IMN complexes. Normal-mode analysis was used to calculate the entropy contributions based on a harmonic approximation after energy minimization of the snapshots. Table 4 represents the calculated results. There are several rigorous methods to calculate binding free energy, such as thermodynamic integration (TI) [Kirkwood, 1935], free energy perturbation (FEP) [Zwanzig, 1954] and linear

interaction energy (LIE) [Aqvist *et al.*, 1994].

`g_mmpbsa` is written in the programming language C and inherits functions from GROMACS [Pronk *et al.*, 2013] as well as APBS [Baker *et al.*, 2001] to calculate energy components given in Eq. (2), except for the entropy and the entropy contribution, which was not included in the binding Energy. If required, the entropic contribution can be calculated by using available modules in the GROMACS package [Pronk *et al.*, 2013]

In MM-PBSA method, the binding free energy (ΔG_{bind}) between a protein (receptor) and a ligand is calculated as follows:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{receptor}} + G_{\text{ligand}}) \quad (1)$$

Each of these free energies can be broken down into following terms:

$$G_i = E_{MM} + G_{sol} - TS \quad (2)$$

i can be the complex, receptor and ligand.

$$E_{MM} = E_{int} + E_{ele} + E_{vdW} \quad (3)$$

$$G_{sol} = G_{pol} + G_{nonpol} \quad (4)$$

Where, E_{MM} , is the gas-phase interaction energy between the protein and the ligand, calculated by summing contributions from the internal energies, E_{int} , (including bond, angle, and torsional angle energies), electrostatic, E_{ele} , and van der Waals, E_{vdW} , interaction energies. G_{pol} and G_{nonpol} are the polar and non-polar contribution to solvation free energy, G_{sol} , respectively, while T is the absolute temperature and S is the solute entropy.

The nonpolar solvation energy, G_{nonpol} , was calculated from the solvent accessible surface area (SASA) using the molsurf program [Connolly, 1983] based on the following equation:

$$G_{nonpol} = \gamma \text{SASA} + \beta \quad (5)$$

where γ is the surface tension, and β is the offset. In our simulations, γ and β parameters were set to $0.0072 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ and 0 kcal mol^{-1} .

The solute entropy, S , is the combination of three components:

$$S = S_{trans} + S_{rot} + S_{vib} \quad (6)$$

where S_{trans} , S_{rot} , and S_{vib} are the contributions related to changes in translational, rotational, and vibrational freedoms, respectively.

One of the unique aspects of this report is binding free energy calculations by MM-PBSA method. There is not any report for this interaction in any literatures by this method. The analysis of energetic contributions to the binding free energy reveals that the average molecular mechanics potential energy in a vacuum $\langle \Delta E_{MM} \rangle$ especially the electrostatics interactions $\langle \Delta E_{ele} \rangle$ are the desired

contributors, as expected. On the other hand, the van der Waals $\langle \Delta E_{vdw} \rangle$ and solvation free energy $\langle \Delta E_{SA} \rangle$ terms are the undesired contributors to binding in all complexes findings that are supported by SASA analysis.

The calculated binding free energies for HSA-IMN1 and HSA-IMN2 complexes are -1024.58 and $-1161.31 \text{ kJ mol}^{-1}$, respectively. With a glance at Table 1, it can be understood that the solvation and entropy terms oppose binding. $\Delta G_{binding}$ values clearly indicate that this binding for IMN2 (subdomain IIA) is energetically more favor than IMN1 (subdomain IB). Thus, it can be deduced that subdomain IIA-IMN complex is more stable than subdomain IB-IMN complex. This finding is in good agreement with the results obtained from the structural analysis.

CONCLUSIONS

This work provided an approach for studying the interaction of IM with HSA by employing calorimetry and MD simulation methods. It demonstrated that the ITC technique of HSA was mainly induced by a static quenching mechanism and the hydrophobic force played a major role in stabilizing the IM-HSA complex, but the presence of hydrogen bonds could not be excluded. Identification of the binding location indicated that the binding of IM to HSA primarily took place in subdomain IIA (site I).

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