Spectroscopic and Dynamic Properties of
Arachidonoyl Serotonin- β -Lactoglobulin Complex:
A Molecular Modeling and Chemometric Study

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Abstract:

UV–Vis absorption data of β -lactoglobulin (BLG) and arachidonoyl serotonin (AA-5HT) in BLG complex were examined and analyzed using chemometrics method. Analysis of the spectral data matrices by using the multivariate curve resolution-alternating least squares (MCR-ALS) algorithm resulted to the pure concentration calculation and spectral profiles resolution of the chemical constituents and the values of $(6.433 \pm 0.019) \times 10^4 \text{ M}^{-1}$, $(4.532 \pm 0.007) \times 10^4 \text{ M}^{-1}$, $(3.364 \pm 0.010) \times 10^4 \text{ M}^{-1}$ and $(2.977 \pm 0.013) \times 10^4 \text{ M}^{-1}$ as estimated equilibrium constants at 288, 293, 298 and 303 K, respectively. The number of chemical constituents involved in the interaction which was extracted by PCA method were free and bound BLG. The spontaneity of the binding process and critical role of hydrogen bonding and van der Waals interactions in stabilizing protein-ligand complex have been designated by negative values of Gibbs free energy, entropy and enthalpy changes. Molecular docking study showed that AA-5HT binds to Val(41), Leu(39), Leu(54), Ile (71), Phe (82), Asn(90), Val(92), Phe(105), Met(107), Glu(108) with the free binding energy of -37.478 kJ/mol . Computational studies predicted that in spite of serotonin (5HT) which anchors to the outer surface of BLG by hydrogen bonds, AA-5HT is situated in the calyx pose and stayed there during the entire time of simulation. This binding is accompanying with no apparent influence on secondary structure and partially destabilization of tertiary structure of BLG which pointed the suitability of this protein as drug carrier for AA-5HT.

Key words: Arachidonoyl Serotonine (AA-5HT), Binding Modes, UV–Vis absorption, Multivariate curve resolution-alternating least squares (MCR-ALS), Molecular Docking, Molecular Dynamics Simulation

1. Introduction

Arachidonoyl serotonin (AA-5HT, Scheme. 1), a synthetic derivative of arachidonic acid, acting as inhibitor of fatty acid amide hydrolase (FAAH; the enzyme responsible for the inactivation of the endocannabinoid anandamide (AEA)), which does not inhibit other carboxylesterases at concentrations lower than 10 μ M, with respect to other FAAH inhibitors developed so far (e.g. URB597, OL-135) [1]. AA-5HT has been denoted as a lipophilic analogue of serotonin with promising applications in biochemistry and drug design. As this compound is not hydrolysed by FAAH, and, hence, does not generate its parent bioactive compounds namely arachidonic acid and serotonin [2], i.p. administration of AA-5HT to rats does not raise serotonin levels in the brain [3]. This latter observation, accompanied by demonstration that AA-5HT forms a rather stable even though not covalent, adduct with the enzyme, implies that this compound can be a highly suitable FAAH inhibitor to be employed *in vivo* [1]. Fascinatingly, AA-5HT is also able to antagonize recombinant vanilloid TRPV1 receptors, with potency slightly higher than that of the widely used TRPV1 antagonist, capsazepine [4]. As a result, AA-5HT is now delineated as a distinctive dual FAAH/TRPV1 blocker [4]. The ability of AA-5HT to adapt simultaneously these two distinct targets might be potentially of importance, as respects the pharmacological or genetic inactivation of FAAH usually cancels out pain and inflammation [5-8], and due to the well-established involvement of TRPV1 receptors in driving inflammatory nociceptive response [9]. This compound seems to be devoid of the main side effects of CB1 agonists and TRPV1 antagonists, and could be used as a template from which new drugs might evolve in the future [10, 11]. Moreover, the role of the endocannabinoid system in appetite stimulation in the adult organism and, perhaps more importantly, its critical involvement in milk ingestion and the survival of the newborn, may not only carry out our understanding of the physiology of food intake and growth, but may also lead to therapeutic applications in wasting diseases and

infant's "failure to thrive" [11-13]. However, this compound is very susceptible to oxidation and photodegradation that limited its applications in pharmacology and food industry.

Bovine β -lactoglobulin (BLG), an abundant milk protein, makes up to 50% of whey and 12% of whole cow milk proteins [14]. It belongs to the family of lipocalin proteins which can bind with many hydrophobic and amphiphilic ligands and drugs such as fatty acids, lipids, aromatic compounds, vitamins, polyethylene glycol and polyamines [15-20]. Spectroscopic analysis of the interaction of BLG with chitosan, with the aim of determining the binding sites of chitosan with BLG and the effects of polymer complexation on the protein stability and conformation, has been revealed that the chitosan binding stabilizes the protein secondary structure and polymer binding did not alter protein conformation [21]. On the other hand, binding to BLG can protect some ligands from oxidation suggesting that BLG could be employed as an effective carrier of oxidation-sensitive drugs [22]. It has been discovered that binding of folic acid to BLG improves its photostability [15]. Also, it has been shown that epigallocatechin-3-gallate (EGCG) in thermally-induced BLG-EGCG conjugates are protected from oxidative degradation [23]. Because of good biocompatibility and biodegradability of milk proteins, BLG could be considered as natural carrier for lipid-soluble drugs [24, 25] and hence would be a candidate for carrying of AA-5HT and related compounds in controlled drug delivery. Moreover, similar conformational changes and ligand binding mechanism between bovine BLG and beta lactoglobulin from other mammals such as goat, indicates that this protein from various sources may have analogous biological role and could be used as drug carrier [26].

With respect to the biological and pharmaceutical importance of AA-5HT as an oxidative sensitive compound, the necessity of suitable biocompatible carrier for improving its bioavailability is inevitable. Here, we reported the complex formation mechanism of BLG-AA-5HT with the aim of evaluate the possible ability of BLG for carrying of this precious

ligand. The involved molecular interactions in the complex formation have been investigated in details by analyzing of spectroscopic titration data using chemometric approach, molecular docking and molecular dynamics simulation techniques.

Scheme. 1. Chemical structure of AA-5HT

2. Materials and methods

2.1. Materials

BLG (variant B > 99% purity) was purchased from Sigma chemical company and used without further purification. AA-5HT (purity not less than 97%, HPLC check) was synthesized in the Laboratory of Oxylipins of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, RAS, Russia as previously described [13] and stored only as ethanol stocks under argon atmosphere at -52°C. UV spectrum of AA-5HT solution in ethanol has a maximum at 282 nm with extinction coefficient of 2790 M⁻¹cm⁻¹. Phosphate buffer (PBS, 10 mM, pH 7.4) was used to keep constant pH value. All used salts for buffer preparation were of analytical grade and were dissolved in deionized water. Ethanol was obtained from Merck Company.

2.2. Preparation of stock solutions

Protein stock solution (5.00 mg/mL) was prepared by dissolving BLG in 10 mM phosphate buffer (PBS), pH 7.4. It was diluted to 1 mg/mL for UV-Vis studies before being

used. The protein concentration was determined spectrophotometrically using the extinction coefficient of $17600 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm [27]. The initial solution of AA-5HT was prepared in ethanol (25 mg/mL) and then diluted with 10 mM PBS for making of ligand stock solution (0.50 mg/mL), the percent of ethanol in this solution was less than 2 v/v and did not have any effect on the conformation of BLG. To avoiding of any possible oxidation, all of the used buffers have been deoxygenized by bubbling of N_2 gas and protected from light.

2.3. UV–Vis spectrophotometry

UV–Vis spectrophotometry was used to determine the thermodynamic parameters of BLG-(AA-5HT) complex formation. Absorption spectra were recorded on a Carry 500 UV–Vis spectrophotometer from 250 to 400 nm. Measurements were carried out in a 1500 μL of protein solution (40 μM) in the cuvette and the stock ligand solution was successively added in μL volumes to the cell placed in the sample chamber (from 0 to 85 μM). Here, PBS, pH 7.4 was used as blank. The absorption spectra were recorded at any specified concentration of ligand. The spectrophotometric titration experiments were run at four different temperatures (288, 293, 298 and 303 K). The resultant data were analyzed using MCR-ALS chemometric method.

2.4. Chemometric analysis

The MCR-ALS is a commonly used technique to resolve the multi-component mixtures into a simple model consisting of a composition-weighted sum of the signals of the pure compounds [28]. This method facilitates decomposition of the multi-component experimental data matrix, **D**, to the pure concentration profiles (**C** matrix) and pure spectral profiles (**S** matrix) of chemical components that simultaneously present in the system, based on a bilinear model as follow, in Eq. 1:

$$\mathbf{D} = \mathbf{CS}^T + \mathbf{E} \quad (1)$$

Here, $\mathbf{E}$ contains the unexplained part of the data by the proposed components or conformations in $\mathbf{C}$ and $\mathbf{S}^T$ ($\mathbf{S}^T$ signifies the transposed of $\mathbf{S}$)

Determination of the number of chemical components in data matrix, $\mathbf{D}$, which is a crucial step in multivariate curve resolution, could be estimated by rank analysis methods, such as singular value decomposition (SVD), principal component analysis (PCA), or some related techniques based on factor analysis, such as evolving factor analysis (EFA) [29] or simple-to-use interactive self-modeling mixture analysis (SIMPLISMA) [30].

In the present study, the recorded UV–Vis spectral data in 1 nm intervals, was exported into MATLAB (Mathworks, Natick, MA, USA) and arranged in a column-wise augmented data matrix with number of titration steps (i.e. the amounts of added ligand) as rows and the number of wavelength (i.e. the different individual spectra collected at various concentration of protein) as columns. PCA technique was applied to identify the number of species in the augmented data matrix. Initial estimates of the concentration profiles and spectra have been taken from SIMPLISMA [31]. The obtained spectral profiles were employed as the initial estimation for the spectral matrix input and iterative alternating least squares optimization of matrices $\mathbf{C}$ and $\mathbf{S}^T$ were carried out until the achievement of convergence. While, MCR–ALS does not necessitate any previous chemical or mathematical knowledge about the system, the addition of information helps to decrease the ambiguity of the final results. Nonnegativity, unimodality (the concentration profiles have a unimodal peak) and the closure constraint in the concentration direction (the sum of the concentrations of the two detected components at different volume ratios of water, namely free and bound BLG is equal to the total concentration of the BLG) were applied during ALS optimization to ensure that the final solution is chemically meaningful.

2.5. Molecular docking

Molecular docking was made with unliganded form of BLG (variant B; PDB ID: 3NPO) (<http://www.rcsb.org/pdb>) as receptor structure, by using AutoDock 4.2 program package. Water molecules were removed from the structure of protein and Gastieger charges and missing hydrogen atoms were added to the BLG in AutoDock Tools environment. The structure of ligand was minimized by the quantum chemistry package Gaussian 03 using Hartree-Fock method and 6-31G(d,p) basis set to get the optimized and stable geometry [32]. The structure with lowest energy was chosen as initial geometry for docking study.

Firstly, a blind docking with grid size of 126×126×126 along X, Y, and Z axes was performed to facilitate the statistical evaluation of possible binding poses of AA-5HT to the BLG. Subsequently, binding locations were defined on the three potential binding sites by a docking box of 60×60×60 dimensions and a grid point spacing of 0.375Å. All docking computations were performed using the AutoDock empirical free energy function and the Lamarckian genetic algorithm with local search [33]. For each docking calculation, a pool of 200 independent conformations generated and assessed with 25,000,000 energy evaluations while the default values were used for all other docking parameters. The resulting docked structures were clustered with a tolerance criterion of 2.0 Å.

2.6. Molecular dynamics simulation

Although, the binding modes of ligands, the binding energy and the residues of protein involved in the interaction with the ligand can be obtained from docking method but the accurate characterization of the structure and dynamics of protein during the binding process cannot be explained by such static docking simulation. Subsequently, the molecular dynamic (MD) simulations carried out with the intention of the main binding mode confirmation and to give the whole impression of the ligand in the binding site of BLG, such as the probable

conformational changes and the stability of complex during the binding. For this purpose, the obtained geometry with the lowest energy from docking was used as starting conformation for MD study.

GROMACS 4.5.4 package with the GROMOS96 43a1 force field [34] was employed to carry out the MD simulations on free BLG and its complex with AA-5HT. The topology parameters of protein were created by using the Gromacs program and the force field parameters for ligand were taken from PRODRG web server [35]. Both free and bound BLG were solvated in a cubic box of the volume of 294 nm³ which contained 9430 single point charge (SPC) [36] water molecules. Given that the negative charge of both systems at physiological pH, the appropriate number of Na⁺ counter-ions were added instead of solvent molecules in the simulation box to make the system electrically neutral and 3D periodic boundary conditions were applied for the BLG in both free and bound states. The energy of two systems was minimized by using the steepest descent method to facilitate the systems to be relaxed and to relieve unfavorable interactions. MD simulation studies comprised of equilibration and production phases. In the first stage of equilibration, a MD simulation of 100 ps was performed in the NVT ensemble to equilibrate the systems at 300 K with the Nose–Hoover thermostat coupling method [37, 38]. In a second part of equilibration, a 100 ps NPT equilibration was performed using the Parrinello–Rahman barostat [39] to keep pressure constant in 1.0 bar. The long range electrostatics was treated with the Particle Mesh Ewald (PME) method [40, 41] using 1.0 nm cut off. For van der Waals interactions, Lennard-Jones potential with 1.0 nm cut off was employed. After equilibration, trajectories with a length of 15 ns were recorded using the leap-frog algorithm for solving the equations of motion [42] with a time step of 2 fs. An analysis of the trajectories (root mean square displacement (RMSD), root mean square fluctuations (RMSF) of atom coordinates and protein structure) was performed using GROMACS analysis tools.

3. Results and discussion

3.1. Thermodynamic properties of binding process using chemometrics

Fig. 1 displays the absorption spectra of BLG in the presence of different concentrations of AA-5HT (Top) and the difference spectrum of BLG with and without ligand (Bottom) at 20 °C (the corresponding plots at the other temperatures are not shown due to space limitation). This plot indicates the enough sensitivity and appropriateness of the UV-vis spectroscopy technique to study the binding of AA-5HT to the BLG. These spectra were used as experimental data for the chemometric analysis to determine the number of total components and to resolve their concentration and spectral profiles.

Fig. 1. (Top) Absorption spectra of BLG in the presence of different concentration of AA-5HT at 20 °C. The arrow indicates the absorption increasing of BLG due to the increasing of

AA-5HT concentration (from 0 to 85 μM). The total concentration of BLG was 40.0 μM .
(Bottom) Difference absorption spectrum of BLG with and without ligand.

The number of significant factors, which indicated the number of chemical constituents involved in the interaction, was extracted by PCA method and the presence of two components in solution was predicted in BLG-(AA-5HT) complex. These two components were free and bound BLG. Then, MCR-ALS method has been used to obtain binding constants and spectral profiles of each component. The initial estimates of spectral profiles were determined using SIMPLISMA method. The obtained concentration and spectrum profiles from results of resolving procedure are shown in Fig. 2.

Fig. 2. (Left) Resolved concentration profiles and **(Right)** the obtained pure spectra of presented components (BLG and BLG-(AA-5HT)) by MCR-ALS procedure at 20 °C.

The spectral changes are attributed to the formation of a 1:1 complex between BLG and ligands as given below:

The binding constant K_b for this equilibrium is represented by the following equation, Eq. 2:

$$K_b = (C_{\text{Complex}} / C^0) / (C_{\text{Ligand}} / C^0)(C_{\text{BLG}} / C^0) \quad (2)$$

Where $C^0 = 1 \text{ mol/dm}^3$. The thermodynamic binding parameters; the standard Gibbs free energy, ΔG^0 , the standard molar enthalpy, ΔH^0 , and the standard molar entropy, ΔS^0 , were calculated to elucidate the energetics of binding process of AA-5HT with BLG. The obtained K_b values, reported in Table. 1, can be used for calculation of Gibbs free energy change of reaction at various temperatures using the relationship, $\Delta G^0 = -RT \ln K_b$, where R and T are gas constant and the absolute temperature, respectively. As the standard enthalpy change does not vary significantly over the considering temperature range, the standard enthalpy and entropy change of binding can be estimated from the following equations, based on the binding constants at different temperatures:

$$\Delta H^0 = d(\Delta G^0 / T) / d(1/T) \quad (3)$$

$$\Delta S^0 = (\Delta H^0 - \Delta G^0) / T \quad (4)$$

Plot of $\Delta G^0/T$ vs. $1/T$ is shown in Fig. 3 and their calculated thermodynamic parameters at different temperatures from 15 to 30 °C are reported in Table. 1. The thermodynamic parameters of binding reaction could be considered to reveal the main driving forces such as hydrophobic, electrostatic, van der Waals interactions and hydrogen bond [43]. The negative value of free energy, ΔG^0 , supports the spontaneity of the binding reaction and relative high affinity of AA-5HT to BLG, and the negative values of ΔH^0 and ΔS^0 representing an enthalpy driven process indicates the importance of hydrogen bonding and van der Waals interactions in solution according to the work of Ross and Subramanian [44]. Therefore, it can be concluded that both hydrogen bonding (may be through OH and NH groups of AA-5HT) and van der Waals interactions play an essential role in the binding of AA-5HT to BLG.

Fig. 3. The variation of $\Delta G^0/T$ vs $1/T$ for binding of AA-5HT to BLG.

Table 1. The calculated thermodynamic parameters for the binding of AA-5HT to BLG.

Temperature (K)	K_b ($10^4 M^{-1}$)	ΔG^o (kJ/mol)	ΔH^o (kJ/mol)	ΔS^o (J/mol.K)
288.0	6.433±0.019	-26.524±0.014	-38.007±1.088	-39.870±0.037
293.0	4.532±0.007	-26.131±0.008	-38.007±1.088	-41.242±0.037
298.0	3.364±0.010	-25.838±0.015	-38.007±1.088	-42.251±0.036
303.0	2.977±0.013	-25.963±0.022	-38.007±1.088	-41.820±0.035

3.2. Protein–ligand interaction results

Three potential binding sites have been reported for ligand binding to BLG: the internal cavity of the β -barrel (calyx), the surface hydrophobic pocket in a groove between the α -helix and the β -barrel, and the outer surface near Trp19-Arg124 [45]. It has been revealed that hydrophobic molecules such as fatty acids, vitamins, particularly vitamin D, retinol and palmitate bind to the central cavity of BLG (calyx) [46, 47], while, polar aromatic compounds, such as p-nitrophenyl phosphate, 5-fluorocytosine, ellipticine, and protoporphyrin, bind to outer surface site [48]. Molecular docking was employed to

understand the binding of AA-5HT with BLG in atomistic details as well as to identify key interactions involved in the complex formation.

A blind molecular docking computation has been done to look for the statistical distribution of binding poses over the whole surface of protein. Clustering of the docked conformations which has been done based on positional root mean square deviation of ligand atoms by Autodock tools, predicted 112 different clusters for binding modes of AA-5HT to the BLG, due to the high flexibility of the hydrophobic chain of the molecule. This flexibility results to have the potential to take on multiple biologically relevant conformations, particularly those in a bent form. The results imparted that AA-5HT docked in the calyx in all of the top-ranked docking poses (20 poses), with binding energies from -38.79 to -29.29 kJ/mol. In the other hand, the percent of possible poses correspond to the second and third experimental binding sites were predicted to be negligible in the blind docking, and AA-5HT preferably binds to the calyx in the most docking runs. Residues identified to interact with AA-5HT, mean free binding energy, and mean binding constant, for docked energies belonging to the top-ranked clusters are listed in Table. 2. In the basis of this table, the estimated binding constant from docking calculation is ≈ 100 times greater than the obtained value from experimental spectroscopic measurements (Table. 1). This difference refers to the inherent inaccuracy of docking scores to predict the binding affinity which refers to inexact description of all molecular interactions. Also, based on Kim and Skolnick study [49] the prediction of the binding affinities of ligand-receptor complex structures appears to be a more difficult task than the prediction of the binding conformations of ligands. Therefore, the docking results would be most informative when this method is applied to a series of ligands to make comparison between their affinities to the specific receptor.

Table. 2. Involved amino acid residues, the mean free binding energy, the related binding constant and the number of H-bonds in BLG-(AA-5HT) and BLG-serotonin interaction.

Molecule	Residues identified to interact with ligand	ΔG° , (kJ/mol)	K_b (10^5 M^{-1})	No. of H-bonds
AA-5HT	Val(41), Leu(39), Leu(54), Ile (71), Phe (82), Asn(90), Val(92), Phe(105), Met(107), Glu(108), Ser(116), Leu(122), Ala(139), Lys(141), Ala(142), Leu(143)	-37.47	37.0	0
Serotonin	Trp(19), Tyr(20), Ser(21), Val(43), Glu(44), Gln(59), Leu(156), Glu(157), Glu(158), His(161)	-33.520	7.50	4

To evaluate the energetics of possible binding modes, three molecular dockings have been conducted with 60×60×60 grid point spaces in the three experimental binding sites of protein. The results revealed this compound binds to the BLG in the first (calyx), second (near the helix) and third binding site with the free binding energy of -38.78, -20.29 and -13.39 kJ/mol, respectively. The binding pose of AA-5HT on BLG with the lowest docked energy belonging to the top-ranked cluster (pose 1) in the first, second and third sites are shown in Fig. 4, Fig. S1 and S2, respectively. In the basis of the blind docking results and energetic evaluation of the binding site, the hydrophobic calyx pocket has been considered as the main binding site of AA-5HT and utilized for further molecular dynamics simulations.

However, in our previous study [25], it was concluded that AA-5HT may bind to the outer surface site of protein as same as serotonin, which is not consistent with our docking results. For that reason, we conducted blind docking of serotonin with BLG to explore its binding site in the protein. Serotonin, on the other hand, did not dock preferentially at the calyx, instead being found at the outer surface site (third site) in the 7/11 of the top-ranked poses with binding energies from -33.72 to -29.25 kJ/mol (Table .2). The binding pose of 5HT on BLG with the lowest docked energy belonging to the top-ranked cluster (pose 1)

predicted from the docking simulations is shown in Fig. 5. There is no any predicted H-bond interactions of AA-5HT with BLG, whereas in serotonin, the polar atoms (N and O) in close contact with the protein was anchored in the binding site by H-bonds with the Val(43), Glu(44), Glu(59), and Glu(157) (Fig. 5), which supplied the different binding site, and subsequently, different driving binding forces of serotonin with respect to the AA-5HT.

Key:

- Non ligand residues involved in the hydrophobic contacts
- Ligand bond

Fig .4. (Right) Three dimensional view of the possible interacting model and binding site, **(Left)** Two-dimensional schematic representation of the main residues involved in the interaction of AA-5HT with BLG in calyx site.

Fig .5. (Right) Three dimensional view of the possible interacting model and binding site, **(Left)** Two-dimensional schematic representation of hydrogen bonds, and the main residues involved in the interaction of serotonin (5HT) with BLG.

Since, ligand docking calculations only predict the binding site, approximate orientation and solvation models, and, cannot determine motion and interactions as a function of time, in the following, unrestrained MD simulations have been applied to provide a means for studying the dynamics and interactions of protein with ligand in the presence of explicit solvent. Therefore, binding pose with the lowest docked energy belonging to the top-ranked cluster (pose 1) was selected for the more accurate docking with 60×60×60 grid and the resultant coordinates was used to molecular dynamics simulation refinements.

3.3. Simulation procedure

The conformation with the lowest energy resulting from accurate docking of the calyx binding site (main binding site of AA-5HT) was selected as the final model for post-docking analysis (15 ns MD simulation). To compare the structural stability of free and ligand bound BLG and determination of the types of interaction involving in the binding reaction, some dynamic structural properties such as root mean square deviations, RMSD, radius of gyration, Rg, hydrogen bonds, root mean square fluctuations, RMSF, and secondary structure have been analyzed. In the following, the detailed calculating results of these parameters are presented.

3.3.1. Root mean square deviations

The trajectory stability for both BLG and complex were evaluated and verified by the analysis of the RMSD of C α atoms of the protein from the initial structure, as function of time. Concerning the Fig. 6 (A), a slight structural re-organization in the first 4000-ps simulation time and short range oscillation in the RMSD of free (blue) and bound BLG (red) around the average value after this time, are observed. In the other word, the RMSD of protein backbone atoms shows the similar trends for both systems indicating the stability and equilibration during 5000 to 15000 ps simulation time.

The mean RMSD values of protein backbone atoms from last 10000 ps trajectory were 0.29 ± 0.02 , and 0.21 ± 0.03 nm for BLG, and BLG-(AA-5HT), respectively. The lower RMSD value of complex showed that binding of the AA-5HT to the BLG decreases the degrees of freedom for protein motions. Also, in order to examine conformational variations of the ligand within the binding site environment, its RMSD of the atomic positions with respect to the starting structure was calculated and shown in Fig. 6 (B). With respect to this figure, the

RMSD value of ligand converged and equilibrated after the 4000 ps, and then fluctuated around the mean value of 0.29 ± 0.02 nm.

Fig. 6. (A) RMSD plots of BLG in free and bound states with AA-5HT. The lower RMSD value in the presence of ligand represents the formation of stable complex. (B) RMSD of ligand in the binding site of BLG with respect to its starting structure.

3.3.2. Radius of gyration

To have a quantitatively measure of the compactness of a structure, the time evolution of the radius of gyration (R_g) can be monitored and regarded as a good assess of the protein collapse dynamics. Hence, R_g for the free and bound form of protein were determined and plotted as a function of time, as shown in Fig. 7. For both free and bound proteins, the R_g values were stabilized at about 5000 ps, indicating that the MD simulation achieved equilibrium after this time. The higher mean value of R_g for complex represented the reducing of compactness and dynamical restriction of the protein due to complexation.

Fig. 7. Gyration radius profiles of free and bound BLG. The increasing of R_g for complex represented the reducing of protein compactness due to complexation.

3.3.3. Hydrogen bond

Hydrogen bond is one of the major forces in ligand binding process which its strength in biological systems varies between 5-30 kJ/mol, and subsequently, they can be formed and broken quickly during binding process, conformational changes, or protein folding. Accordingly, hydrogen bonds in biological conditions may be switched on or off with energies that are within the range of thermal fluctuations. This is one of the main factors that facilitates macromolecular association events, and biological activity. The number and stability of H-bonds between protein and ligand is an important factor contributing to the stability of ligand binding.

The presence of H-bonds between protein and AA-5HT was calculated every 5 ps using the `g_hbond` module of GROMACS within the last 8 ns of the simulation time. It is found that the number of H-bonds ranged from 0 to 4 with the average number of 1.5, during this time as shown in Fig. 8. The y-axis of the plot was selected to 2 as the H-Bonds with values of greater than 2 are found only sporadically. This MD result has not been achieved by

docking calculations which predicted the lack of any H-bond and exclusive participation of hydrophobic interactions in the binding process. These H-bonds have been formed with Pro38, Phe82, Asn90, and Gln120 of BLG in the simulation time. The H-bonds Asn90···OAV, Phe82···NBO, Pro38···NAV, and, Asn90···NBO (ligand atom types assignment has been displayed in Fig. 9) vanished at the initial steps of the analyzing trajectory, while the strong H-bonds between the Gln120 and ligand (NAV and OAV atoms) were formed at approximately 9600 ps and conserved in the rest of the simulation time, which implied the slightly rotation of AA-5HT in the calyx. Based on docking studies (Fig. 4), AA-5HT docks in the calyx in all of the top-ranked docking poses. With respect to Fig. 9, that has visualized the binding pose in the last step of MD simulation, the ligand remained in the calyx binding site during the simulation time but in comparison to the initial docking pose, AA-5HT rotated and formed H-bonds with its neighbor residues (Fig. 9) which implies the importance of H-bond interaction in the binding of this ligand to BLG. This is consistent to the experimental spectroscopic results.

Fig. 8. Formation and breakage of intermolecular H-bonds during the ligand-binding (AA-5HT with BLG) process.

Fig. 9. (Right) Three dimensional view of the possible interacting model and binding site, **(Left)** Two-dimensional schematic representation of the main residues involved in the interaction of AA-5HT with BLG in the last frame of the molecular dynamics simulation showed the formation of H-bond and supplied the importance of hydrogen bonds interactions in binding process.

3.3.4. Root mean square fluctuations

In order to evaluate the local protein mobility, the time-averaged RMSF values of free and bound BLG were calculated. The results were plotted against residue numbers at the simulation trajectory and have been shown in Fig. 10. Analysis of fluctuation score revealed

the presence of higher degree of flexibility in free BLG in comparison to the bound protein representing that binding of AA-5HT restricted the conformational space explored by BLG. In the other word, overall reduction in the RMSF values, in the case of BLG complex, reveals that binding of ligand induced constrains in the flexibility of protein structure. The particular regions that directly in contact with ligand (pointed in Fig. 10) showed more significant reduction of the RMSF that is related to intermolecular interactions of these residues with the ligand and slightly reorientation of the AA-5HT in the binding pocket with respect to the docking pose.

Fig. 10. RMSF of BLG residues respect to their time-averaged positions for free and bound BLG. RMSF reductions in regions that directly in contact with ligands are pointed with arrows.

3.3.5. Secondary structure

Additional information on the structural changes of protein during the binding process is obtained by the analysis of time-dependent secondary structure fluctuations. The evaluation of changes in the secondary structure during the simulation time can lead us to obtain precious information regarding protein stability. The secondary structure of BLG before and

after the binding was calculated with the DSSP code [50]. The program generated Fig. 11 that represents the secondary structure contents of free and bound BLG during the simulation. The results indicate that the more important secondary structure contents, beta-sheets and alpha-helices, were constant throughout the simulation period for both free and bound protein which signifies not only the stability of free protein trajectory during the simulation time, but also, the insignificant change of secondary structure due to the binding of AA-5HT. Furthermore, in our previous work [25] it has been proved by CD spectroscopy, this ligand has no apparent influence on BLG secondary structure, however it partially destabilizes the tertiary structure of protein, which is in agreement with our MD simulation results.

Fig. 11. Variation of the secondary structure of protein versus time for free (**top**) and bound (**bottom**) BLG during the 15 ns simulation

4. Conclusions

Binding of AA-5HT as unique dual FAAH/TRPV1 blocker and new drugs template candidate has been considered with BLG and studied using chemometric analysis of UV-Vis spectroscopic data, molecular docking and molecular dynamics simulation. Negative sign of both enthalpy and entropy changes acquired by the chemometric analysis of the UV-Vis spectrophotometric data bolded the importance of both hydrogen bonds and van der Waals interactions in the binding process. Molecular docking calculations resulted that in spite of serotonin which binds to the outer surface site, AA-5HT binds to the hydrophobic pocket, calyx, on the basis of hydrophobic interactions of its lipophilic long chain with the calyx residues, namely, Val, Leu, Ile, Phe, Asn, Met, Glu, and, Gln. 112 resulting clusters of binding modes found by cluster analysis of blind docking refers to high structural flexibility of this arachidonic acid based ligand. More accurate docking with 60×60×60 docking box, manifested the exclusive participation of hydrophobic forces to the binding interactions.

The RMSD convergence of trajectory together with the R_g profile from MD simulations established the stability of BLG-ligand complex and validity of docking results. Secondary structures plotted by DSSP code of GROMACS represented insignificant changes in the secondary structure of BLG due to the binding of AA-5HT. The RMSF profiles of free and bound BLG residues and H-bond analysis reveal slight movement of ligand from its docking pose during the MD simulation. The experimental and computational results represent the binding of AA-5HT in the calyx binding site of BLG with relatively high affinity and minor changes in structure and dynamics of the protein. Therefore, BLG might act as suitable carrier for transporting of this pharmaceutically important compound *in vitro*.

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