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Review Article

Fatty acid binding to serum albumin: Molecular simulation approaches

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Abstract

Background: Binding affinity for human serum albumin (HSA) is one of the most important factors affecting the distribution and free blood concentration of many ligands. The effect of fatty acids (FAs) on HSA-ligand binding has long been studied. Since the elucidation of the 3-dimensional structure of HSA, molecular simulation approaches have been applied to studies of the structure-function relationship of HSA-FA binding.

Scope of review: We review current insights into the effects of FA binding on HSA, focusing on the biophysical insights obtained using molecular simulation approaches such as docking, molecular dynamics (MD), and binding free energy calculations.

Major conclusions: Possible conformational changes on binding of FA molecules to HSA have been observed through MD simulations. High- and low-affinity FA-binding sites on HSA have been identified based on binding free energy calculations. The relationship between the warfarin binding affinity of HSA and FA molecules has been clarified based on the results of simulations of multi-site FA binding that cannot be experimentally observed.

General significance: Molecular simulation approaches have great potentials to provide detailed biophysical insights into HSA as well as the effects of the binding of FAs or other ligands to HSA.

Key words

human serum albumin; fatty acid; simulation; molecular dynamics; free energy; docking

1. Introduction

Human serum albumin (HSA) is the most abundant protein in blood plasma and has ligand-binding and enzymatic properties. HSA is a transporter and depot protein for numerous endogenous compounds (e.g., fatty acids [FAs]) and exogenous compounds, and is also capable of binding to many commonly used drugs. Binding to HSA is one of the factors influencing drug disposition [1-3]. Recently, interactions between HSA and environmentally hazardous substances such as carbon nanoparticles [4] and PCB153 [5] have been also reported.

Since the first report of the 3-dimensional structure of HSA in 1992 [6], more than 70 such structures of HSA have been deposited in the Protein Data Bank (PDB). X-ray crystallography [6-30] and nuclear magnetic resonance (NMR) spectroscopy [31, 32] have revealed not only the structure of HSA but also those of its ligand-binding modes. Based on these structures, additional significant insights, such as the dynamic properties of HSA, have been revealed using molecular simulation approaches. Molecular dynamics (MD) simulation is a well-established method for the analysis of macromolecular conformations, especially focusing on the dynamic nature of macromolecules. Currently, MD simulations are playing a larger role in the study of macromolecules as a result of continuous improvements in algorithms, software and hardware [33-35]. In this review, we describe current insights into the conformation and function of HSA obtained with molecular simulation approaches, such as MD, molecular docking, and binding free energy calculations, focusing on the effects of FA binding.

2. FA binding to HSA: experimental approaches

2.1 Interaction between HSA and FA

FAs play critical roles in energy metabolism and the synthesis of membrane phospholipids. In the body, FAs are transported via the lymphatic and vascular systems. Owing to their low solubility in water, FAs require a transporter to increase their concentration in vascular and interstitial compartments. HSA is the main FA-binding protein in extracellular fluid [36]. Under normal physiological conditions, HSA binds with approximately 0.1–2 mol FA per mole protein [37]. The FA/HSA molar ratio increases to 6 during fasting or maximum exercise [38, 39] or under pathological conditions such as diabetes [40, 41] and cardiovascular disease [42].

Interaction between HSA and FAs has long been studied. Early affinity constants of HSA reported for FAs indicated that multiple FA-binding sites exist on HSA [43-47]. Later, the presence of 7 FA-binding sites on the protein was elucidated through X-ray crystallographic studies (Figure 1). These FA-binding sites are common for medium-/long-chain or monosaturated/polysaturated FAs [7, 10, 11, 48]. The FA-binding affinity (high/low) of each site has been also identified with ^{13}C NMR spectroscopy [49, 50] and site-directed mutagenesis of HSA [51] (Figure 1). Comparison of the 3-dimensional structures of defatted HSA and HSA-FA complexes has revealed that the binding of FA molecules to HSA causes a relative rearrangement at the I-II and II-III domain interfaces [7, 10, 52] and conformational changes of the side chains of subdomain IIA [17].

2.2 Effect of FA on HSA-ligand binding

Ligand-binding affinity for HSA is among the most important factors affecting the distribution and free concentration of many ligands, and the binding affinity is likely to be influenced by the binding of FAs to HSA, because some of the FA-binding sites overlap with ligand-binding sites [17]. Table 1 shows the ligands that bind to FA-binding sites, which have been revealed by X-ray crystallography. FA-binding site 7 (subdomain IIA), and sites 3 and 4 (subdomain IIIA) are known as major drug-binding sites I and II, respectively [17, 53]. In

general, bulky heterocyclic anions bind preferentially to FA-binding site 7, while sites 3 and 4 are preferred by aromatic carboxylates with an extended conformation [52, 54]. FA-binding site 1 is also a major ligand-binding site, especially for endogenous compounds such as heme [13, 15], bilirubin [21], and prostaglandins [23]. The binding of heme to this site has been reported to reduce the affinity of ligands for drug-binding site I [52]. Few ligands have been reported that bind to FA-binding sites 2, 5, and 6. Sites 5 and 6 have been identified as the sites with highest and low FA affinities, respectively [50]. The binding of FA to site 2, as well as the binding of heme to site 1, has been reported to stabilize the rotated conformation of domain I relative to domain II [7, 10, 52, 55]. Details on an expected correlation between preferred binding sites and classes of bound ligands are well summarized in recent reviews [3, 52, 54]. Numerous experimental studies have also indicated that ligand-binding affinity to HSA can be modulated through simultaneous binding of FAs [55-68]. The modulation is caused by competitive binding between a ligand and an FA at the same binding site [17] or allosteric effects from the binding of FAs [62, 66-68].

3. Molecular simulation of HSA and its application to HSA-FA binding studies

3.1 Molecular simulation studies of HSA to date

X-ray crystallography and NMR spectroscopy have made significant contributions to the structural analyses of HSA. In addition to these experimental techniques, molecular simulation approaches have become feasible for further structural-functional analyses of HSA. In this section, we review docking, MD, and binding free energy calculation studies of HSA.

3.1.1 Molecular modeling studies using electrostatic potential calculation or molecular docking simulations

The electrostatic potential around HSA has been analyzed using the determined HSA structure to find ligand-binding sites on HSA and bound conformations of ligands. Grymonpré et al. [69] have predicted the binding site of hyaluronic acid from the calculated electrostatic potential around HSA. Song and Gunner [70] have analyzed the binding of chloride ions using multi-conformation continuum electrostatics.

Molecular docking approaches have been widely used for the molecular modeling of HSA-ligand binding. Although more than 70 HSA structures on PDB database have given insights into ligand-binding sites on HSA and bound conformations of many ligands [3, 52, 54], it is still difficult to predict the exact binding modes (ligand-binding site and bound conformation) of unknown HSA-ligand complexes, because of the existence of multiple ligand-binding sites on HSA and flexible amino acid side chains at those sites. Docking simulations have been performed to estimate HSA-ligand binding modes computationally. Currently, more than 60 HSA-ligand docking studies have been reported (Table 2). The binding modes obtained in these studies can help further structural-functional analyses of HSA.

The results of docking simulation are generally reported along with experimental studies such as equilibrium dialysis, circular dichroism, fluorometry, calorimetry, and spectroscopy [5, 63, 67, 71-124]. In spite of this, choosing the correct target for docking can sometimes be difficult owing to the existence of multiple ligand-binding sites on HSA [3, 17, 54]. Hence, the results of such studies should be interpreted with caution. In the case of bilirubin, for example, docking simulation has been carried out for subdomain IIA (drug-binding site I) of HSA [125]. Although the study reported that bilirubin was docked to subdomain IIA in a robust manner, X-ray crystallography identified that bilirubin binds to subdomain IB instead [21]. Many of the experimental studies have examined only subdomains IIA and IIIA (drug-binding site II) as target sites for docking, because these sites are primary drug-binding

sites [53]. Experiments that examine the binding of a ligand at sites other than drug-binding sites I and II may be useful to select the correct site for docking simulations.

3.1.2 MD simulations for analyzing conformations of HSA or HSA-ligand complexes

The first MD simulation of HSA was reported in 2001, and it analyzed the influence of the protonation states of Lys195 and Lys199 on HSA conformation [126]. Simulations of the binding of divalent cations (Co^{2+}, Cu^{2+}, Ni^{2+} and Cd^{2+}) to the N-terminus of HSA were reported in 2004 [127]. These simulations used part of the HSA molecule. The MD simulation of the whole HSA molecule was first published in 2005 and reported that inter-domain motion of the unliganded HSA molecule was observed in a 2-ns MD simulation [128]. Fujiwara and Amisaki [129] later carried out 10-ns MD simulations of defatted and FA-bound HSA to analyze the conformational changes of HSA brought about by the binding of FA molecules, as described in section 3.2.1. The interaction of HSA with macromolecules has also been analyzed using MD simulations with a chrysotile surface [130], a carbon nanotube surface [131], self-assembling monolayers [132], poly(amidoamine) dendrimers [133], and HSA-HSA adsorption [134].

Three-dimensional structures of HSA obtained with X-ray crystallography are used in common for starting structures for MD simulations. HSA-ligand structures obtained through docking simulations have been also used for MD to confirm the stability of docked complexes [80, 84, 87, 90, 100, 104, 119, 120, 135-138]. Some of the HSA-ligand structures obtained using docking and MD simulations have been applied to quantum mechanics calculations of the excitation energies [138] and quantitative structure-activity relationship analyses of HSA-ligand binding affinity [139]. In addition, longer time scale (≥100 ns) MD simulations have been recently performed for analyses of the HSA-aspirin complex [140], the HSA-heme complex [141], the structural role of disulfide bridges in HSA [142], and the conformational flexibility of the unliganded HSA [119]. Thus, MD simulations are playing an increasingly important role in structural-functional studies of HSA.

3.1.3 HSA-ligand binding free energy calculations based on MD trajectory data

An MD trajectory of ligand-bound structures is a collection of estimated equilibrium conformations. Trajectory data have been used for the calculation of HSA-ligand binding free energy as well as conformational analyses. The HSA-ligand binding free energy (binding affinity) correlates with percentage plasma protein binding [143]. To date, calculated binding free energies have been reported for Gd-AAZTA complex (AAZTA = 6-amino-6-methylperhydro-1,4-diazepine tetraacetic acid) [144], zidovudine and its derivatives [63], FAs (see section 3.2.2) [145], levamlodipine [76], flavones [78], perfluorooctanoic acid and perfluorooctane sulfonate [146], mexiletine [110], hydroxyquinoline molecules [119], PCB153 [5], and warfarin [147]. Fujiwara and Amisaki [147] have calculated binding free energies under various FA/HSA molar ratios, as described in section 3.2.3. In these reports, calculated binding affinities were consistent with those from experimental approaches, indicating the appropriateness of the calculations.

3.2 Applications of molecular simulations to HSA-FA binding

As of Febrary 2013, eight studies have been published that report the application of molecular simulations to HSA-FA binding. In this section, we review these published studies concerning (1) conformational changes of HSA caused by FA binding [129], (2) conformation and binding affinity of an FA molecule at each FA binding site [145, 148], and (3) effect of FA binding on HSA-ligand interaction [63, 72, 144, 147, 149].

3.2.1 MD studies of conformational changes in HSA caused by binding of FA molecules

Fujiwara and Amisaki [129] carried out conformational analyses of the unliganded HSA and the HSA-FA complex with 10-ns MD simulations. The radius of gyration of MD simulations of the unliganded HSA was almost the same that of the experimental value, indicating that the equilibrium state of HSA molecules in aqueous solution was reproduced well in the MD simulations. The main differences between the unliganded HSA and the HSA-FA complex were observed in the primary internal motions characterized by the first 3 principal components at domains I and III (Figure 2). The directional motion projected on the first principal component of the unliganded HSA was conserved in the HSA-FA complex as the third principal directional motion with higher frequency. Thus, their MD study provides insights into the possible conformational changes of HSA caused by the binding of FA molecules on a scale of 10-ns. A method to obtain a full impression of the conformational freedom of HSA is to perform simulations over longer time periods. Continuing improvements in MD algorithms and software, and enhanced hardware performance will enable longer MD simulations, which may provide further insights into the effect of FA binding on the conformation of HSA.

3.2.2. Identification of high-affinity FA binding sites on HSA by molecular simulations

When considering the interaction between FA molecules and other ligands, the identification of high-affinity FA-binding sites on HSA is very important (see section 2.2). Seven possible FA-binding sites have been revealed with X-ray crystallography [10, 11]. High- and low-affinity FA binding sites have also been experimentally determined with ^{13}C NMR spectroscopy [49, 50].

Rizzuti et al. [148] have analyzed the structural basis of high-affinity FA-binding site 5 using MD simulations. They observed that Lys525 was important because the residue anchored FA head-groups. Fujiwara and Amisaki [145] have quantitatively examined the HSA-FA affinity at each FA-binding site using MD simulations and binding free energy calculations. The calculated value of each absolute binding free energy deviated greatly from the experimental binding free energies as estimated using the HSA-FA affinity constants [45] (Figure 3). However, the spectrum of the affinity (high/low) over FA binding sites was successfully identified. They identified FA-binding sites 5, 4, and 2 as high-affinity sites, and 1, 3, 6, and 7 as low-affinity sites, identical to those of the experimental approaches [50] (see Figure 1). Binding free energy calculation may be useful for comparison of the relative stabilities of HSA-ligand complexes, although the accurate calculation of absolute binding free energy is one of the challenges to be tackled in theoretical studies.

3.2.3. Effect of FA molecules on HSA-ligand binding

The published X-ray structures of HSA-ligand-FA complexes have given insights into the effect of FA on HSA-ligand binding [17, 54]. However, the number of the structures is not nearly large enough to cover the binding modes of the complexes, because of the existence of multiple ligand-binding sites on HSA and flexible amino acid chains at those sites. As the second best approach, molecular simulations have been performed to analyze the effect of FA molecules on HSA-ligand binding. One of the advantages of molecular simulation approaches is that conditions that are not observed experimentally can be simulated computationally. The effects of FA molecules on the interaction between HSA and ligands have been analyzed through docking simulations. Paal and Shkarupin [149] have reported reduced binding affinities of paclitaxel, in comparison to the defatted HSA, at paclitaxel-binding sites on the HSA-FA complex. Gianolio et al. [144] have observed that Gd-AAZTA binds with different affinities to defatted (low affinity) and FA-bound HSA (high affinity) as a consequence of the conformational changes upon FA binding. Fanali et al. [72] have performed docking analyses of 3 anti-HIV drugs in 4 of the 7 FA-binding sites to compare intermolecular energies of the

drugs at each site. Quevedo et al. [63] have observed that reduced affinities of zidovudine derivatives in the presence of FAs were caused by an intense electrostatic repulsion between FA and ligands with negative charges.

An approach focusing on the number of FA molecules bound to HSA has been also reported. Fujiwara and Amisaki [147] have analyzed the relationship between HSA-warfarin binding affinity and the positions of bound FA molecules. Based on the affinity at each FA-binding site (section 3.2.2), they constructed 11 "virtual" HSA-warfarin-FA complexes, each with different FA molecule positions. These virtual complexes were used for MD simulations and binding free energy calculations for HSA-warfarin binding (Figure 4). The results indicate that unfavorable steric effects on HSA-warfarin binding affinity (in terms of the van der Waals energy contribution) were caused by the binding of an FA molecule to FA-binding site 2, which is closest to the warfarin-binding site (see Figure 1). Conversely, the magnitude of HSA-warfarin binding free energy was discovered to be largest (i.e., the HSA-warfarin binding affinity was strongest) when 3 FA molecules were bound to the high-affinity sites. The relationship between HSA-warfarin binding affinity and the number of bound FA molecules (Figure 4) coincided with the previous observations [59]. This study clarified the structural and energetic properties of these steric/allosteric effects of FAs on HSA-warfarin binding affinity. The molecular simulation approach described above may be applicable to binding studies of interactions between other ligands and HSA.

4. Conclusions

We reviewed recent molecular simulation studies to analyze the structure-function relationship of HSA, focusing on the HSA-FA binding. Differences in the directional motions of domains I and III between the unliganded HSA and the HSA-FA complex have been analyzed with MD simulations (see section 3.2.1). High- and low-affinity FA-binding sites on HSA have been identified quantitatively with MD simulations and binding free energy calculations (see section 3.2.2). In addition, HSA-ligand binding free energies were calculated with respect to the positions of FA molecules bound to HSA (see section 3.2.3). Such approaches will continue to evolve in themselves in terms of simulation theory and computer technology. For example, MD studies on millisecond time scales are now available [150]. To date, MD simulations on nanosecond time scales ($\geq$100 ns) have been reported for HSA. Longer-time-scale MD calculations may elucidate unknown conformational characteristics of HSA.

One of the characteristics of HSA-FA binding is the binding of multiple FA molecules. We expect that HSA is useful as a model of multiple-ligand-binding proteins, and that additional advances in molecular simulation approaches may lead to the elucidation of the relationship between conformation and function of HSA.

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Table 1. Ligands bound to FA-binding sites on human serum albumin (HSA).

Site [a]	Bound ligand identified by X-ray crystallography
1	Azapropazone [17], AZT[b] [20], bilirubin-IXα [21], dansyl-L-asparagine [26], dansyl-L-arginine [26], dansyl-L-glutamate [26], fusidic acid [21], heme [13, 15], indomethacin [17], iophenoxic acid [27], naproxen [19], Δ^{12}-prostaglandin J_2 [23], salicylic acid [18, 20], triiodobenzoic acid [7]
2	Halothane [9]
3, 4	CMPF[c] [17], dansyl-L-asparagine [26], dansyl-L-norvaline [26], dansyl-L-phenylalanine [26], dansyl-L-sarcosine [26], diazepam [17], diflunisal [17], halothane [9], ibuprofen [17], indoxyl sulfate [17], iophenoxic acid [27], propofol [9]
5	Fusidic acid [21], oxyphenbutazone [17], propofol [9]
6	DAUDA[d] [29], diflunisal [17], halothane [9], ibuprofen [17]
7	Aspirin [18], azapropazone [17], AZT[b] [20], citric acid [16], CMPF[c] [17], dansyl-L-arginine [26], dansyl-L-asparagine [26], dansyl-L-glutamate [26], dansyl-L-phenylalanine [26], DAUDA[d] [29], diflunisal [17], halothane [9], indomethacin [17], indoxyl sulfate [17], iodipamide [17], iophenoxic acid [27], lysophosphatidylethanolamine [22], oxyphenbutazone [17], phenylbutazone [17], salicylic acid [18, 20], triiodobenzoic acid [7], warfarin [12, 17]

[a] The numbering of the FA binding sites was sourced from Bhattacharya et al. [10].
[b] 3'-Azido-3'-deoxythymidine.
[c] 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid.
[d] 11-(Dansylamino) undecanoic acid.

Table 2. HSA-ligand docking simulations reported as of Febrary 2013.

Year	Ligand docked to HSA
2006	Formononetin [71]
2007	Anti-HIV drugs (abacavir, nevirapine, atazanavir) [72], bilirubin [125], eupatilin [73], paclitaxel [149],
2008	Noble gas [151], zidovudine and its derivatives [63]
2009	Daunorubicin [74], deoxyuridine [75], levamlodipine [76],
2010	Angiotensin II receptor blockers [136], anti-Parkinson's disease drugs (apomorphine and benserazide) [77], flavones [78], flavonoids [67], glucose [135], metsulfuron-methyl [79], perfluorooctanoic acid and perfluorooctane sulfonate [146], β-sitosterol [80], sulfometuron-methyl [152]
2011	Anthraquinone dye [81], cyclophosphamide hydrochloride and aspirin [82], diazepam and ∆9-tetrahydrocannabinol [83], hen egg white lysozyme and triacetylchitotrioside [84], isoniazid and rifampicin [85], lomefloxacin [86], titanocene [153], warfarin (docked to methylglyoxal-modified HSA) [87]
2012	2-aminobenzothiazole [88], amlodipine and propranolol [89], amodiaquine [90], anthraquinone dye [91], anti-breast cancer drugs (fluoxymesterone, cyclophosphamide) [92], benzoxazole [93], betulinic acid [137], bilirubin [94], catechin [95], chlorpyrifos [96], daphnin [97], daunorubicin analog [98], ethyl maltol [99], extrinsic fluorescent probe [100], flavins [101], flavokawain B [102], guaijaverin [103], harmalol [104], hesperidin [105], 10-hydroxycamptothecin [106], merocyanine 540 [107], 6-mercaptopurine [108], metalaxyl [109], mexiletine [110], nitrofurazone [111], phthalate plasticizers [112], rhaponticin [113], virstatin [114], water soluble copper(II) complex [115]
2013	Acridine yellow and proflavin [138], [2,2'-bipyridyl]-3,3'-diol [116], demeclocycline [117], furosemide [118], hydroxyquinoline derivatives [119], PCB153 [5], Schiff base complex [120], strictosamide [121], tetracycline hydrochloride [122], tyramine-based anthraquinone analogue [123], water-soluble tungstenocene derivatives [124]

Figure legends

Figure 1. Ribbon model of the human serum albumin (HSA)-palmitate complex derived from X-ray crystallography (PDB ID: 1E7H). HSA is composed of 3 homologous domains, I-III, each is divided into subdomains A and B. The 7 palmitate molecules are shown in blue (identified as high-affinity fatty acid [FA] binding sites) or yellow (identified as low-affinity FA-binding sites) in a space-filling representation [50]. The numbering of the FA binding sites was sourced from Bhattacharya et al. [10]. Molecular graphics images were prepared with VMD (version 1.9.1) [154].

Figure 2. Directional motions of the unliganded HSA and the HSA-FA complex projected on the first, second, and third principal components (PCs 1-3). The arrows in the figure indicate the approximate directions of cooperative motions of C_α atoms. The directional motion projected on PC1 of the unliganded HSA is similar to that projected on PC3 of the HSA-FA complex. Molecular graphics images were prepared with VMD (version 1.9.1) [154]. This figure was reproduced and adapted from Fig. 8 of PROTEINS: Structure, Function, and Bioinformatics 64 (2006) 730-739 [129].

Figure 3. Relationship between experimental and calculated HSA-FA (myristate, palmitate) binding free energy for 3 high-affinity FA binding sites (sites 5, 4, 2). Affinity constants (K_1, K_2, K_3) were taken from Ashbrook et al. [45]. The experimental binding free energy ($\Delta G_{bind,expt}$) was calculated using the equation $\Delta G_{bind,expt} = -RT \ln K$, where R and T are the gas constant and the absolute temperature, respectively. The calculated values of absolute binding free energies deviated considerably from the experimental binding free energies (red lines). This figure was reproduced and adapted from Fig. 4 of Biophysical Journal 64 (2008) 95-103 [145], with permission from Elsevier.

Figure 4. Four of the 11 "virtual" HSA-warfarin-myristate complexes and the relationship between calculated HSA-warfarin binding free energies (ΔG_{bind}) and the number of bound FA (myristate) molecules. Based on molecular dynamics simulations of the 11 virtual HSA-warfarin-myristate complexes, ΔG_{bind} was calculated for each complex. In the graph, the position of bound FA molecules is also indicated. This figure was reproduced in part with permission from Chemical and Pharmaceutical Bulletin Vol. 59 No.7 [147]. Copyright 2011 The Pharmaceutical Society of Japan.

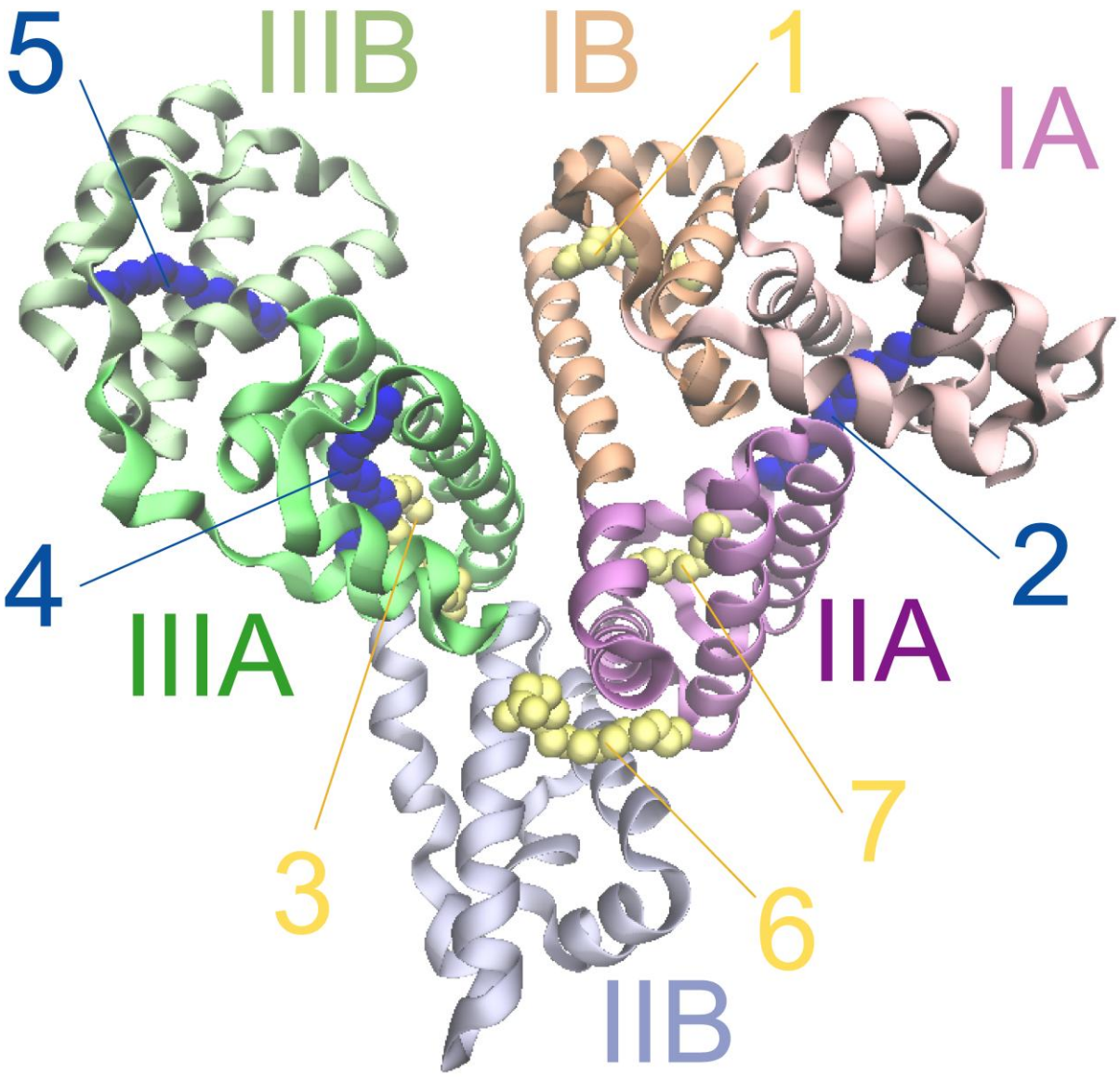

Figure 1

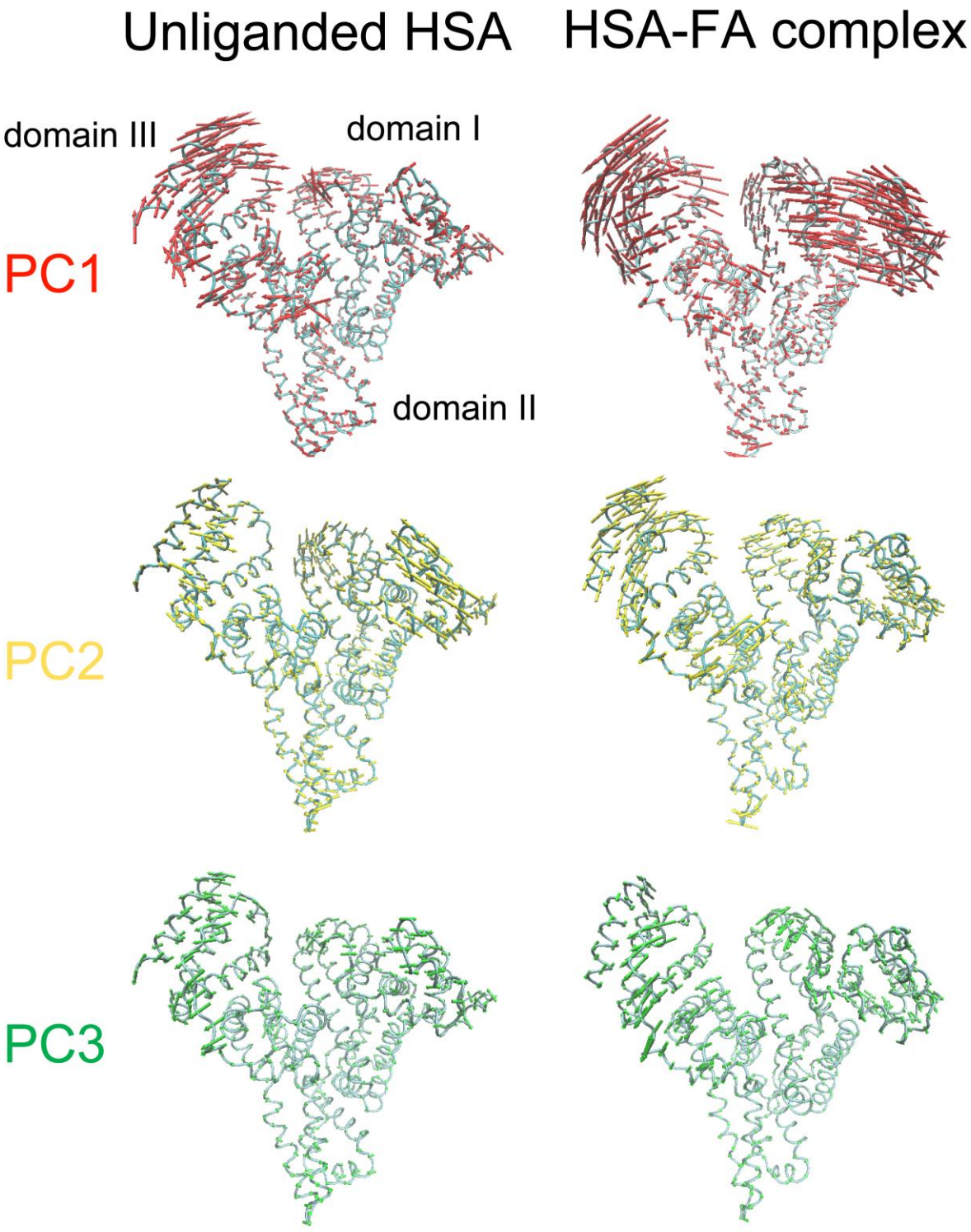

Figure 2

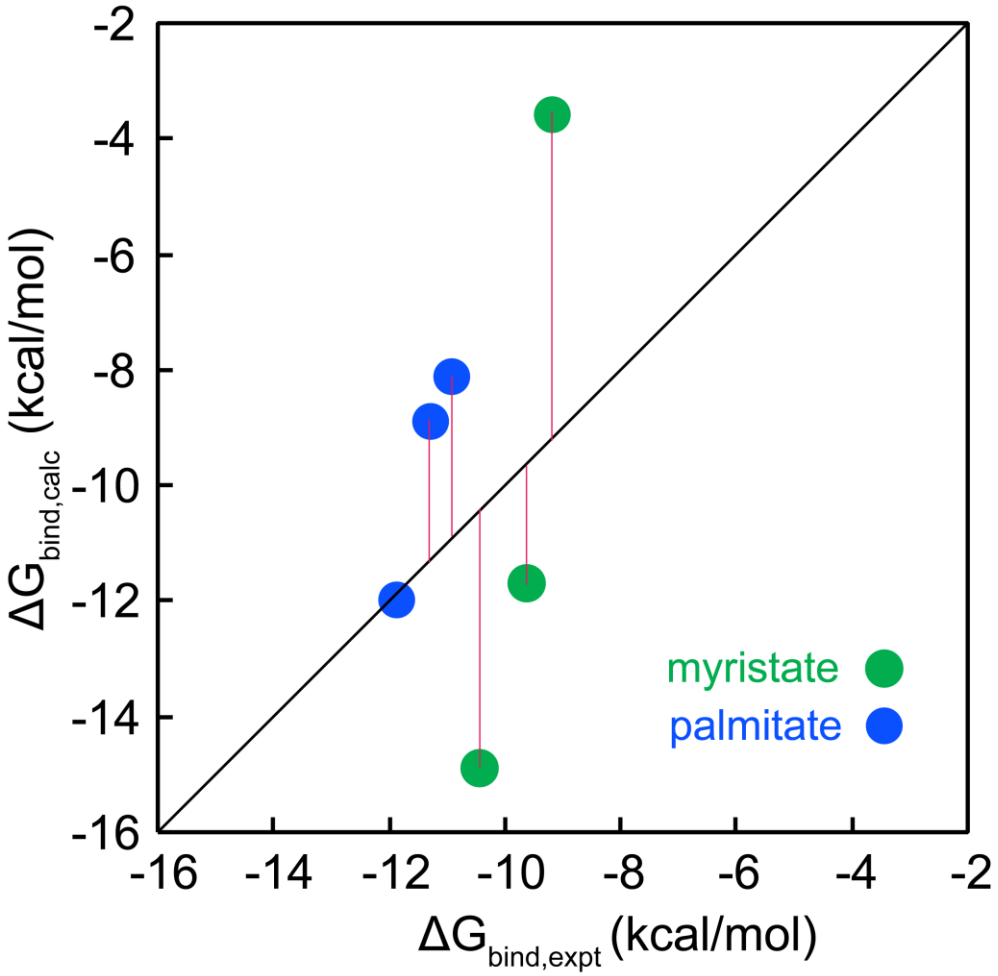

Figure 3

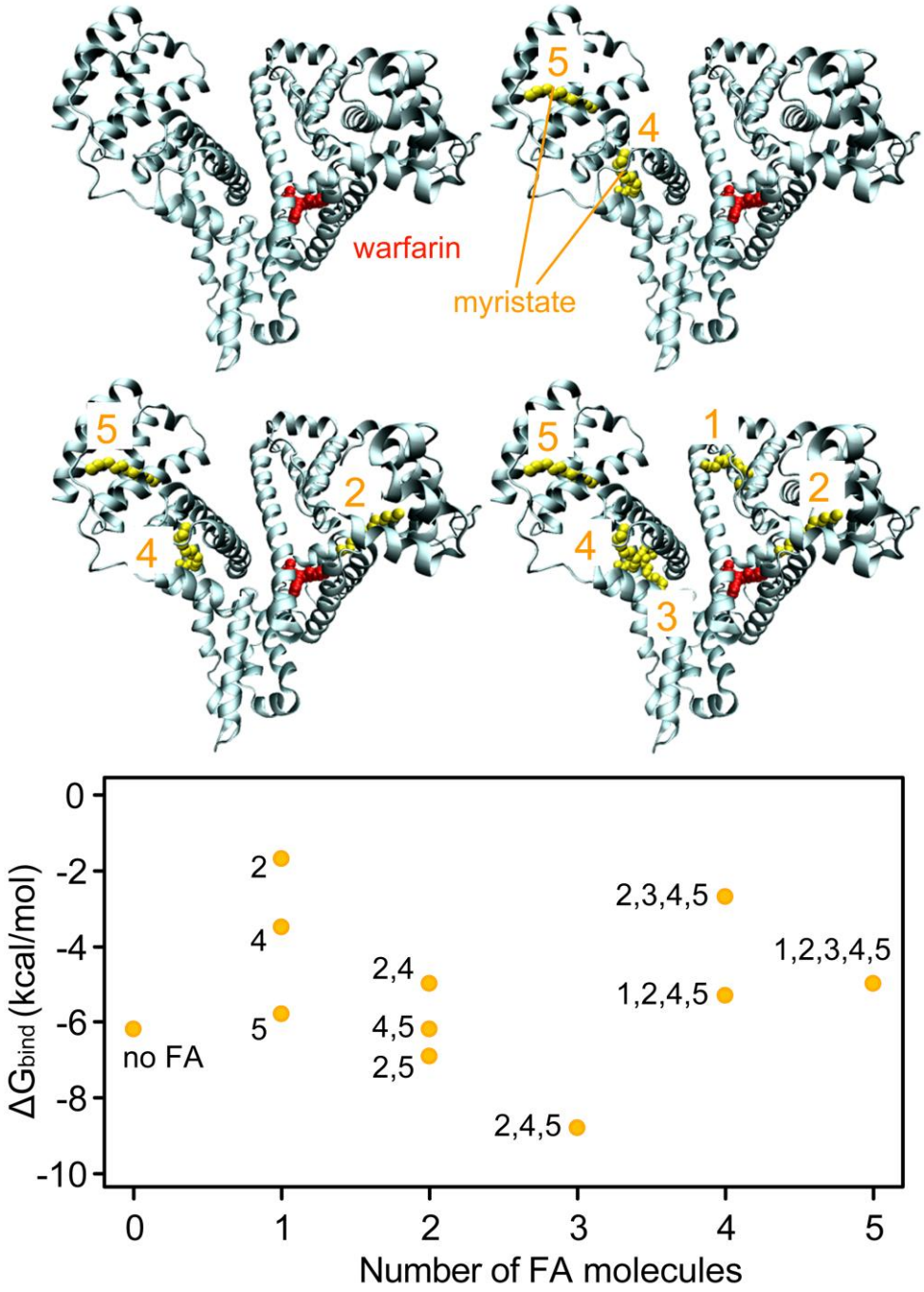
warfarin
myristate
5
4
5
2
4
5
1
2
4
3
0
-2
-4
-6
-8
-10
ΔGbind (kcal/mol)
no FA
2
4
5
2,4
4,5
2,5
2,4,5
2,3,4,5
1,2,4,5
1,2,3,4,5
0
1
2
3
4
5
Number of FA molecules

Figure 4

Highlights

- Binding of FA molecules to HSA can modulate ligand binding affinity to HSA.
- Molecular simulation approaches have been applied to structural analyses of HSA.
- Possible conformational changes of HSA-FA binding were analyzed by MD simulations.
- Binding free energy calculations identified high/low affinity FA binding sites.
- Molecular simulation analyzes conditions that cannot be experimentally observed.