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Review

Allostery in a monomeric protein: The case of human serum albumin

Paolo Ascenzi [a,b,*], Mauro Fasano [c]

[a] Department of Biology and Interdepartmental Laboratory for Electron Microscopy, University Roma Tre, Rome, Italy
[b] National Institute for Infectious Diseases I.R.C.C.S. "Lazzaro Spallanzani", Rome, Italy
[c] Department of Structural and Functional Biology, and Center of Neuroscience, University of Insubria, Busto Arsizio (VA), Italy

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ABSTRACT

Human serum albumin (HSA), the most prominent protein in plasma, binds different classes of ligands at multiple sites. The globular domain structural organization of monomeric HSA is at the root of its allosteric properties which are reminiscent of those of multimeric proteins. Here, both functional and structural aspects of the allosteric modulation of heme and drug (e.g., warfarin and ibuprofen) binding to HSA and of the drug-dependent reactivity of HSA-heme are reviewed.

Contents

1. Introduction

Human serum albumin (HSA), the most abundant protein in plasma, is characterized by an extraordinary ligand binding capacity providing a depot and carrier for many compounds; consequently, it affects pharmacokinetics of many drugs, provides the metabolic modification of some ligands, renders potential toxins harmless transporting them to disposal sites, accounts for most of the antioxidant capacity of human serum, and displays (pseudo-) enzymatic properties [1–17].

HSA is constituted by a single non-glycosylated all-α chain of 65 kDa arranged in a globular heart-shaped conformation containing three homologous domains (labeled I, II, and III). Each domain is made up by two separate helical subdomains (named A and B), connected by random coils. Terminal regions of sequential domains contribute to the formation of inter-domain, 9-turn-long helices linking domain IB to IIA (residues 173-205), and IIB to IIIA (residues 336-398), respectively (Fig. 1). Despite their structural similarity, each domain interacts with the neighbor domain(s) in different ways. Therefore,

Abbreviations: FA, fatty acid; HDL, high density lipoproteins; HPX, hemopexin; HPX-heme, heme-hemopexin; HSA, human serum albumin; HSA-heme, human serum heme-albumin; HSA-heme-Fe(II), ferrous HSA-heme; HSA-heme(II)-NO, ferrous nitrosylated HSA-heme; HSA-heme(III), ferric HSA-heme; HSA-heme(III)-NO, ferric nitrosylated HSA-heme; LDL, low density lipoproteins.

* Corresponding author. Department of Biology, University Roma Tre, Viale Guglielmo Marconi 446, 00146 Rome, Italy. Tel.: +39 06 5733 3494; fax: +39 06 5733 6321.
E-mail address: ascenzi@uniroma3.it (P. Ascenzi).

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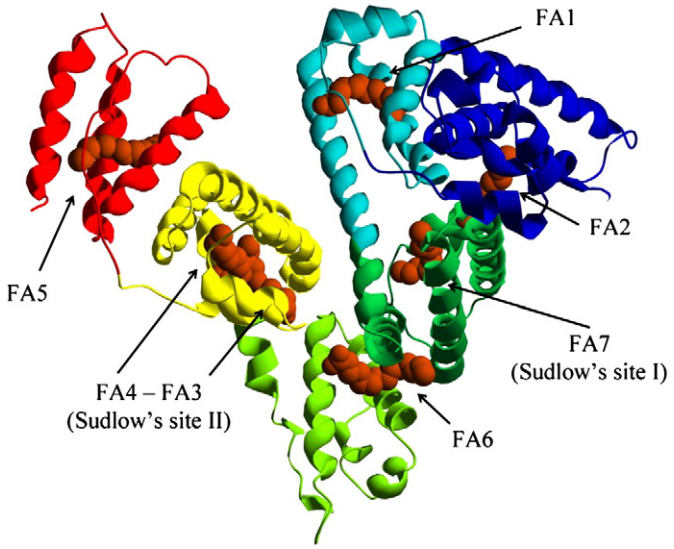


Fig. 1. The human HSA structure. HSA is rendered with ribbons colored as follows: subdomain IA: blue; subdomain IB: cyan; subdomain IIA: green; subdomain IIB: yellow-green; subdomain IIIA: yellow; subdomain IIIB: red. Myristate ions are rendered as spacefill in brown. FA and drug binding clefts (FA1 to FA7, and Sudlow's sites I and II, respectively) are labeled. Atomic coordinates have been retrived from PDB entries 1O9X and 1BKE [21,34]. The picture was drawn with Swiss-PDB-Viewer [93].

the orientation of domains I–II with respect to domains II–III constitutes a highly asymmetric environment where a variety of ligand binding sites is distributed (Fig. 1) (see [3,5,11,17]).

2. HSA displays multiple binding sites

HSA is able to bind up to seven equivalents of long chain fatty acids (FAs; which represent the primary physiological ligands) at multiple binding sites (labeled FA1 to FA7; see Fig. 1) with different affinity. Thus, HSA acts as a carrier and depot for FAs released from adipose tissue following triacylglycerol hydrolysis [18]. Site-directed mutagenesis studies have revealed that sites FA2, FA4, and FA5 have the highest affinity for FAs [19,20]. These sites, together with FA1 and FA3, share the presence of a basic/polar group at the entrance of the cavity that stabilize the carboxylate head of the FA. FA1, FA3, FA4, and FA5 clefts are usually pre-formed, in contrast FA2 forms as a consequence of a rigid-body rotation of domain I with respect to domain II. In particular, FA1 is a cavity within subdomain IB contacting the IB-IIA polypeptide linker; FA2 is located at the interface between subdomains IA, IB, and IIA; FA3 and FA4 both contribute to the formation of a large cavity in subdomain IIIA; FA5 is located within subdomain IIIB with the polar head orientated towards subdomain IIIA. On the other hand, FA6 is a solvent-accessible linear slot located at the interface between subdomains IIA and IIB, and FA7 is a large flattened cavity comprised within subdomain IIA. FAs accommodate into FA6 and FA7 as a consequence of hydrophobic interactions only [12,17,21–26].

FA binding sites also provide accommodation for several endogenous and exogenous ligands. In particular, a wide variety of drugs displays appreciable affinity for one or more binding sites of HSA [11–13,17]. This issue is of great relevance as binding of HSA improves drug solubility and plasma half-life, but at the same time reduces the free, active concentration of drugs [13,17]. Early investigations by Sudlow and coworkers lead to the identification of two main drug-binding sites (Fig. 1). Bulky heterocyclic anions bind preferentially to Sudlow's site I (corresponding to a large cavity comprising FA7), whereas Sudlow's site II (overlapping FA3 and FA4) is preferred by aromatic carboxylates with an extended conformation. Remarkably, warfarin and ibuprofen are the prototypical ligands of Sudlow's site I and II, respectively [1–3,5,12,17,21,24,27–31].

2.1. Sudlow's site I is the warfarin binding cleft

In defatted HSA, Sudlow's site I is a pre-formed binding pocket (encompassing FA7) within the core of subdomain IIA that comprises all six helices of the subdomain and a loop-helix feature (residues Tyr148-Leu154) contributed by IB; the entrance to Sudlow's site I is restricted by subdomain IIIA. The prevalence of basic residues and the absence of acidic amino acid side chains define the specificity of the pocket for molecules with two anionic features on opposite sides that can simultaneously interact with the two polar patches. Indeed, although the interior of the pocket is predominantly apolar, it contains two clusters of polar residues, an inner one towards the bottom of the pocket (Tyr150, His242, and Arg257) and an outer cluster at the pocket entrance (Lys195, Lys199, Arg218, and Arg222). The large binding cavity is comprised of a central zone from which three distinct compartments extend. The back end of the pocket is divided by Ile264 into left and right hydrophobic sub-chambers, whereas a third sub-chamber protrudes from the front of the pocket, delineated by Phe211, Trp214, Ala215, and Leu238 and aliphatic portions of Lys199 and Arg218 (Fig. 2) [12,17,24].

Drugs (e.g., warfarin) cluster in the center of Sudlow's site I, having a planar group pinned snugly between the apolar side-chains of Leu238 and Ala291. In contrast, there is much greater variation in the drug position within the plane perpendicular to the line between these two residues. Compounds occupy the apolar compartments of Sudlow's site I to different extents, e.g. warfarin occupies the right-hand and the front sub-chambers. All of the compounds are positioned to make a hydrogen bond interaction with the hydroxyl group of Tyr150, this residue assuming a central role in drug interactions. The *R*-(+) and *S*-(−) enantiomers of warfarin bind in essentially the same position as one another and appear capable of making a total of three hydrogen bonds with Tyr150, His242, and either Lys199 or Arg222. The similarity of the binding environments for the enantiomers helps to explain the poor stereoselectivity of HSA for warfarin [12,17,24].

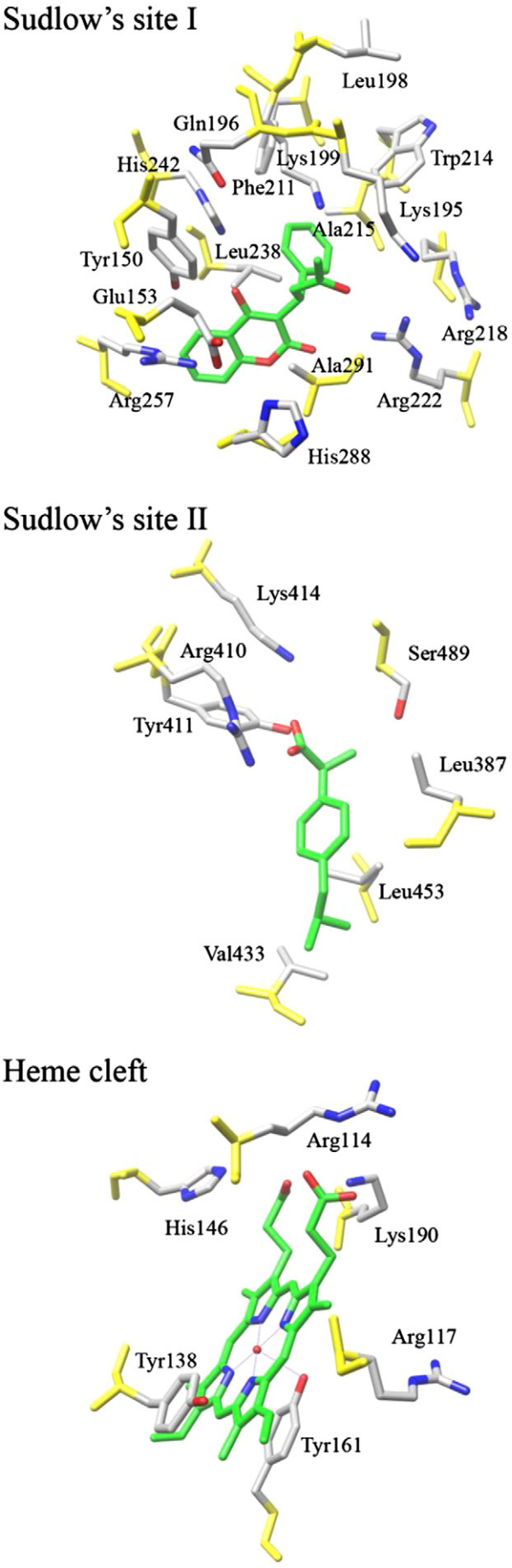
Superposition of the HSA-drug complexes reveals that there are only small side-chain movements associated with binding of drugs having Mr ≤ 310 Da. In contrast, thyroxine (Mr = 777 Da) binding to HSA induces the dislocation of Tyr150 and Trp214 [12,17,31].

In the presence of FAs, Tyr150 from subdomain IB moves to interact with the carboxylate moiety of the lipid. This helps to drive the relative rotation of domains I and II and induces an extensive rearrangement of the H-bond network involving Tyr150, Glu153, Gln196, His242, Arg257, and His288, increasing the volume of Sudlow's site I and altering its polarity distribution. The helix containing Leu198 is also displaced outwards inducing twisting of the adjacent helix from subdomain IIIA (residues Glu442-Lys466) [12,17].

In spite of the structural changes induced by FAs binding, many of the features that emerged from the comparison of complexes of Sudlow's site I drugs with defatted HSA are also evident in the presence of myristate. Indeed, the ligands bind in the central portion of the cavity, pinned between Leu238 and Ala291. Nevertheless, since Tyr150 is removed from the pocket to interact with the FA, it is no longer available to make the central contribution to drug binding that is observed in complexes with defatted HSA. Rather, different drugs make use of the various basic and polar ligands on both sides of the binding pocket. Interactions with solvent, as observed for warfarin, may also help to compensate for the loss of Tyr150 [12,17,24,30,31].

2.2. Sudlow's site II is the ibuprofen binding cleft

Sudlow's site II (overlapping FA3 and FA4) is composed of all six helices of subdomain IIIA and is topologically similar to Sudlow's site I (subdomain IIA), although smaller. Sudlow's site I comprises a largely pre-formed hydrophobic cavity, as observed in Sudlow's site I, even if significant differences in the polar features of the two drug binding



pockets occur. The principal binding region corresponds to the central portion of Sudlow's site I and appears to possess just one sub-compartment, the rear right-hand hydrophobic sub-chamber, though in this case the sub-chamber is only accessed following ligand-induced side-chain movements. Furthermore, the entrance to Sudlow's site II is more exposed to the solvent. In contrast to Sudlow's site I, Sudlow's site II has a single polar patch, located close to one side of the entrance of the binding pocket and centered on Tyr411 but also including Arg410, Lys414, and Ser489. Thus, differences in shape, size, and polarity help to account for the different binding specificities of Sudlow's sites I and II (Fig. 2) [12,17].

Drugs (e.g., ibuprofen) cluster in the center of Sudlow's site II, interacting with the hydroxyl group of Tyr411; Arg410 and Ser489 residues also contribute salt-bridge and hydrogen-bond interactions to drug binding. Thus, the presence of a single basic polar patch located at one end of the apolar binding pocket is at the root of the specificity for drugs with a peripherally located electronegative group (Fig. 2) [12,17].

As observed for drug binding to Sudlow's site I, there is little side-chain movement associated with binding of drugs (e.g., ibuprofen) with Mr ranging between 197 and 250 Da). Arg410 and Val433 are the most susceptible residues to ligand-induced alterations. Binding of diazepam, which has a larger, branched structure (Mr = 285 Da) is accompanied by rotations of the Leu387 and Leu453 side-chains allowing the phenyl ring of the drug to access the rear right hand sub-chamber of the pocket. As in Sudlow's site I, variations in the water structure may help to make the pocket more adaptable [12,17].

Further evidence of the adaptability of Sudlow's site II derives from the fact that although it appears to be relatively small, it can bind two molecules of long-chain FAs (in sites FA3 and FA4). The methylene tails of FAs bound to FA3 and the polar patch interacts with the carboxylate moiety of FAs bound to FA4 [12,17].

Compounds that bind to Sudlow's site II may also interact with additional HSA clefts outside subdomain IIIA. There is crystallographic evidence for ibuprofen binding to the FA6 pocket [12,17]. Additionally, a third binding site (possibly FA2) has been suggested on the basis of solution studies addressing the effect of ibuprofen on heme affinity and heme-HSA spectroscopic properties and reactivity [29,59,60].

2.3. FA1 is the heme binding cleft

The FA1 binding site displays a dramatic versatility, thus adopting different conformations to accommodate different ligands. In general, the site opens to the ligand by unstacking two tyrosine (Tyr138 and Tyr161) residues located at the center of a D-shaped cavity that is limited by hydrophobic side-chains of subdomain IB all around and is open to the solvent facing subdomain IIIA. FA1 does not appear to be a high-affinity FA binding site, rather it has been proposed that it has evolved to selectively bind the heme, Tyr138 and Tyr161 residues providing π–π stacking interactions with the porphyrin and supplying a donor oxygen (from Tyr161) to the heme-Fe(III)-atom. Heme is secured to HSA by the long IA–IB connecting loop that fits into the cleft opening. Heme propionates point toward the interface between domains I and III and are stabilized by salt bridges with Arg114, His146, and Lys190; remarkably, Arg117, that stabilizes the carboxylate head of FA in FA1, is not implicated in heme binding. The enclosure of heme in a hydrophobic cavity is of course reminiscent of the construction of the oxygen-binding proteins myoglobin and

Fig. 2. Schematic representation of Sudlow's site I (FA7; top), Sudlow's site II (FA3-FA4; middle), and the heme cleft (FA1; bottom) of HSA. Warfarin and ibuprofen are bound to Sudlow's sites I and II, respectively. Sudlow's sites I and II and the heme cleft are presented in the same orientation as Fig. 1. Drug carbon atoms are colored in green and backbone atoms of the residues building up the sites are colored in yellow. Atomic coordinates have been retrived from PDB entries 2BXD, 2BXG, and 1N5U [12,33]. The picture was drawn with Chimera [94].

hemoglobin and has raised the possibility of exploiting HSA-heme-Fe (II) as an artificial oxygen carrier (Fig. 2) [17,19,20,29,32–35]. It should be noticed that bilirubin, the breakdown product of heme, binds to the same cleft in a completely different mode. Indeed, the unstacking of Tyr138 and Tyr161 is not needed for bilirubin binding to FA1 [17,36].

FA1 is crucial for heme scavenging by HSA, providing protection against free heme oxidative damage, limiting the access of pathogens to heme, and contributing to iron homeostasis by recycling the heme iron. In fact, during the first seconds after heme appearance in plasma, more than 80% of this powerful oxidizer binds to high density lipoproteins (HDL) and low density lipoproteins (LDL), and only the remaining 20% binds to HSA and hemopexin (HPX). Then, HSA and HPX remove most of the heme from HDL and LDL. Afterwards, heme transits from HSA to HPX, that releases it into hepatic parenchymal cells after internalization of the HPX-heme complex by CD91 receptor-mediated endocytosis. HDL and LDL, the most oxidatively intolerant plasma components, bind the heme with the highest affinity, however kinetics of heme transfer from HDL and LDL to HSA and HPX is faster than the heme-induced lipoprotein oxidation [3,9,37–47].

Lastly, heme binding confers to HSA spectroscopic and reactivity properties, thus providing a suitable tool to investigate allosteric and competitive properties [14,16,29,32,44,48–53].

3. Ferric heme binding to HSA is modulated allosterically

Ferric heme (heme-Fe(III)) binds to HSA by a simple equilibrium, values of the dissociation equilibrium constant (i.e., K ($=k_{off}/k_{on}$)), of the second-order association rate constant (i.e., k_{on}), and of the first-order dissociation rate constant (i.e., k_{off}) being 1.3×10^{-8} M, 7.4×10^{5} M^{-1} s^{-1}, and 9.6×10^{-3} s^{-1}, respectively, at pH 7.0 and 25.0 °C [10,48].

Heme-Fe(III) binding to HSA induces a distortion of the long helix that connects domain I and II, with the consequent reorientation of amino acid side chains that form ligand binding cavities in domains I and II (Fig. 3) (see ref. [17]). Consequently, heme-Fe(III) binding to HSA is allosterically modulated by ligands and vice versa [10,13,14,16,20,29,32–35,48,54–56]. Saturating concentrations of drug (e.g., warfarin) binding to Sudlow's site I (encompassing FA7) decrease by about one order of magnitude both values of K and k_{on} for heme-Fe(III) binding to HSA. According to linked functions [57,58], heme-Fe(III) inhibits drug binding to Sudlow's site I by the same extent. Indeed, the affinity of ligands for Sudlow's site I is reduced by about one order of magnitude upon heme-Fe(III) binding to HSA. In contrast, values of the first-order rate constant for the dissociation of heme-Fe(III) and of Sudlow's site I ligands are insensitive to third component(s) (i.e., Sudlow's site I ligands and heme-Fe(III), respectively) [10,12–14,20].

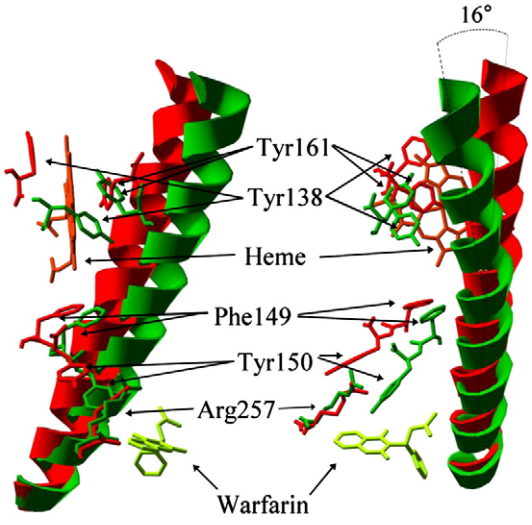


Fig. 3. The ligand-dependent conformational transition of HSA. Heme-bound (red) and warfarin-bound (green) conformations of HSA display different orientation of residues forming warfarin (pale green) and heme (orange) binding regions, respectively. The long interdomain helix connecting domains I and II is rendered as ribbon. The tilting by 16° of the interdomain helix is highlighted. The two pictures are rotated approximately of 90°. Atomic coordinates have been retrived from PDB entries 1O9X and 2BXD [12,34]. The picture was drawn with Swiss-PDB-Viewer [93].

The observed functional link between the heme site (i.e., FA1) and the warfarin cleft (i.e., FA7) has been confirmed in the truncated form of HSA, encompassing residues Asp1-Glu382 [52]. Interestingly, this truncated HSA derivative lacks Sudlow's site II (located in subdomain IIIA; i.e., the primary ibuprofen cleft). Nevertheless, ibuprofen binding exerts a negative allosteric effect on heme-Fe(III) binding, and vice versa, thus showing that FA1, the heme binding cleft, and FA6, the secondary ibuprofen binding site, are functionally linked. In fact, the K value for heme-Fe(III) binding to the Asp1-Glu382 HSA mutant increases from 7.4×10^{-8} M, in the absence of FA6 ligands, to 3.4×10^{-6} M, in the presence of saturating amounts of ibuprofen. Accordingly, ibuprofen affinity for the FA6 site decreases by about one order of magnitude upon heme-Fe(III) binding to the Asp1-Glu382 HSA mutant. Eventually, a third, weak ibuprofen binding site (possibly FA2) could be taken into account in order to explain the observed variations in heme-Fe(III) affinity and heme-Fe(III)-HSA spectroscopic properties and reactivity that occur at high ibuprofen concentrations. Indeed, in the 10^{-3}–10^{-2} M ibuprofen concentration range a stabilization of the HSA conformation endowed with high heme affinity was observed, together with a distorted heme-Fe(III) geometry. As a consequence, facilitated $^{\bullet}$NO dissociation from HSA-heme-Fe(II)-NO and impaired peroxynitrite isomerization by HSA-heme-Fe(III) were reported [29,52,59,60].

4. HSA-heme reactivity is modulated allosterically

HSA-heme displays heme-based reactivity [11,16,28,44,49,50,54,56,59–68]. Ferrous HSA-heme (HSA-heme-Fe(II)) binds reversibly CO and $^{\bullet}$NO and facilitates detoxification of nitrogen reactive species [16,44,49,63,66–72]. Although O_2 binding to wild type HSA-heme-Fe(II) is impaired by O_2-induced oxidation of the heme-Fe-atom, artificial HSA-heme-Fe(II) mutants that bind reversibly O_2 and CO could be of relevant clinical importance not only as red cell substitutes but also as O_2-providing therapeutic reagents [66,72–74].

$^{\bullet}$NO dissociation from ferrous nitrosylated HSA-heme (HSA-heme-Fe(II)-NO) and detoxification of nitrogen reactive species by HSA-heme-Fe(II)-NO and ferric HSA-heme (HSA-heme-Fe(III)) are allosterically-modulated by drugs binding to FA6 and FA7 (e.g., abacavir, warfarin, and ibuprofen) [59,60,67,68].

$^{\bullet}$NO binds reversibly to HSA-heme-Fe(II) and ferrous HSA-tetraphenylporphinatoiron [68,70,71,73], values of kinetic and thermodynamic parameters (i.e., $h_{on}\sim1\times10^{7}$ M^{-1} s^{-1}, $h_{off}\sim1\times10^{-4}$ s^{-1}, and $H\sim1\times10^{-11}$ M) being similar to those reported for ferrous sperm whale myoglobin nitrosylation [71,75].

In the absence of drugs, the value of h_{off} for $^{\bullet}$NO dissociation from penta-coordinate HSA-heme(II)-NO is 1.3×10^{-4} s^{-1}. Abacavir and warfarin facilitate $^{\bullet}$NO dissociation from HSA-heme-Fe(II)-NO, the h_{off} value increasing to 8.6×10^{-4} s^{-1} in the presence of saturating amounts of these drugs. From the dependence of h_{off} on the drug concentration, values of the dissociation equilibrium constant for the abacavir and warfarin binding to HSA-heme-Fe(II)-NO (i.e., $L=1.2\times10^{-3}$ M and 6.2×10^{-5} M, respectively) were determined. The drug-dependent increase of h_{off} reflects abacavir and warfarin binding to Sudlow's site I, inducing the stabilization of the six-coordinate derivative of the HSA-heme-Fe(II)-NO atom [48,68]. Although no direct structural evidences are available, the observed behavior has been ascribed to the pivotal role of Tyr150, a residue that

either provides a polar environment in Sudlow's site I or protrudes into the FA1 cleft, depending on the occupation of both sites [48,68].

Also ibuprofen facilitates $^{\bullet}$NO dissociation from HSA-heme-Fe(II)-NO, in fact the h_{off} value increases from 1.3×10^{-4} s^{-1}, in the absence of the drug, to 9.5×10^{-3} s^{-1}, in the presence of 1.0×10^{-2} M ibuprofen. The analysis of the dependence of h_{off} on the drug concentration allowed the determination of values of the dissociation equilibrium constants for ibuprofen binding to HSA-heme-Fe(II)-NO ($L_1=3.1\times10^{-7}$ M, $L_2=1.7\times10^{-4}$ M, and $L_3=2.2\times10^{-3}$ M). The drug-dependent increase of h_{off} reflects ibuprofen binding to the FA3-FA4 cleft (Sudlow's site II), to the FA6 site, and possibly to the FA2 pocket [29,48,59,60]. Although the absence of crystallographic data does not allow to propose a precise mechanism, ibuprofen binding drives profound changes in both local and global HSA-heme structure, triggering the hexa-coordination of the HSA-heme-Fe(II)-NO atom. In this context, it has been proposed that His146 reorientation would provide the nitrogen donor atom for the sixth (axial) heme-Fe(II)-NO coordination position [29,59].

HSA-heme(II)-NO and HSA-heme(III) catalyze peroxynitrite scavenging. These (pseudo-)enzymatic reactions are modulated allosterically by abacavir and ibuprofen [60,67].

Mixing of HSA-heme(II)-NO and peroxynitrite solutions leads to HSA-heme(III) by way of ferric nitrosylated HSA-heme (HSA-heme(III)-NO), according to (Scheme 1) [67]:

$$\text{HSA}-\text{heme(II)}-\text{NO} + \text{HOONO} \xrightarrow{b_{on}} \text{HSA}-\text{heme(III)}-\text{NO} \xrightarrow{d} \text{HSA}-\text{heme(III)} + {}^{\bullet}\text{NO} + {}^{\bullet}\text{NO}_2 + \text{H}_2\text{O}$$

(Scheme 1)

Values of b_{on} and d for peroxynitrite-mediated oxidation of HSA-heme(II)-NO are 6.5×10^{3} M^{-1} s^{-1} and 1.8×10^{-1} s^{-1}, respectively [67]. Remarkably, abacavir induces the enhancement of the b_{on} value to 3.6×10^{5} M^{-1} s^{-1} without affecting the d value [67]. The effect of abacavir on $^{\bullet}$NO/peroxynitrite scavenging by HSA-heme(II) reflects drug binding to Sudlow's site I and the concomitant drug-induced hexa-coordination of the heme-Fe-atom of HSA-heme(II)-NO by Tyr161 [11,13,14] (see below). Note that the formation of the ferric form of heme-proteins is facilitated by binding of Tyr residue(s) and phenolate (derivatives) to the heme-Fe atom [76,77].

HSA-heme-Fe(III) catalyzes peroxynitrite isomerization, according to (Scheme 2):

$$\text{HSA}-\text{heme}-\text{Fe(III)} + \text{HOONO} \xrightarrow{f_{on}} \text{HSA}-\text{heme}-\text{Fe(III)}-\text{OONO} \xrightarrow{fast} \text{HSA}-\text{heme}-\text{Fe(III)} + \text{NO}_3^- + \text{H}^+$$

(Scheme 2)

The f_{on} value for peroxynitrite isomerization by HSA-heme-Fe(III) is 4.1×10^{5} M^{-1} s^{-1}. The HSA-heme-Fe(III)-catalyzed isomerization of peroxynitrite has been ascribed to the reactive penta-coordinated heme-Fe(III) atom. Ibuprofen impairs allosterically peroxynitrite isomerization by HSA-heme-Fe(III), the value of f_{on} lowering to 3.5×10^{4} M^{-1} s^{-1}. Again, ibuprofen binding to FA2, the only ligand binding site that provides contacts with different HSA subdomains (being located at the interface between subdomains IIA and IIB), may be postulated as responsible to induce hexa-coordination of the heme-Fe(III) atom lowering the metal center reactivity [29,60].

5. Structural bases of the allosteric control

The allosteric regulation of HSA action is essentially based on crystallographic investigations concerning HSA(-FA)(-ligand) complexes (see ref. [17]). However, since the heme (i) binds to the FA1 site in a shape overlapping the periphery of the macrocycle and (ii) displays some interactions with HSA similar to those of myristate, some functional considerations applying to long-chain FAs could apply also to the allosteric modulation of heme binding to HSA and HSA-heme reactivity. Crystallographic evidences (see ref. [17]) are substantiated by solution studies (see ref. [29]) indicating that the allosteric modulation of heme binding to HSA and of HSA-heme reactivity by third components reflects changes of the coordination state of the heme-Fe-atom.

FA binding to HSA induces a conformational transition where a tilt in interdomain helices drives a rotation of domains I and III by about 16° with respect to domain II (Fig. 3). Crystallographic and solution evidences indicate that the observed conformational transition is not determined by the highest affinity binding events, rather it cooperatively takes place when the protein is half-saturated. Noteworthy, two medium-affinity sites (i.e., FA2 and FA3) are located at domain interfaces and therefore may drive the conformational transition observed upon FAs binding [21–24,32,51,78].

The FA2 site is formed by two separate sub-sites, one hosting the methyl end of the hydrophobic tail in domain I and the other accommodating the polar head in subdomain IIA. The half-sites line up as a consequence of the 16° rotation of domain I with respect to domain II. In FA3 the carboxylate group of the FA interacts with Arg348 by displacing Glu450 and causing a local distortion of subdomain IIIA. As a consequence, Asp451 rotates toward domain I and forms a salt bridge with Lys195, thus helping the rigid-body rotation of domains I and III with respect to domain II [17,21–24]. Although there are no crystallographic evidences to date, it has been assumed that the FA-loaded conformational state of HSA corresponds to the basic (B) state of the protein [16]. The physiological meaning of this conformational transition is not currently known, however it is reasonable to imagine that it triggers a mechanism of receptor-mediated endocytosis to deliver FAs to hepatocytes. Interestingly, HSA could also deliver heme by the same mechanism [35].

FAs are effective in the regulation of heme binding to HSA, both by direct competition and allosteric mechanisms. Sudlow's site I (i.e., FA7) ligands (e.g., warfarin) have a higher affinity for the FA-free conformation, whereas FA1 ligands (e.g., heme) have a higher affinity for the FA-bound conformation, FA1 and FA7 sites being functionally coupled (see above) [13,14,16,20,52]. A mechanistic explanation would involve the occupation of FA2 as it forces the 16° rotation of domain I with respect to domain II. Actually, binding of myristate to FA2 attracts Tyr150 and Arg252, two key residues positioned in the center of the apolar chamber of Sudlow's site I, towards the carboxylate moiety of the FA [14,17,20]. On the other hand, the reorientation of Tyr150 drives the interaction of Phe149 with heme, thus making a stabilizing π–π interaction and explaining the allosteric modulation observed in solution studies [13,14,16,17]. This allosteric regulation is not observed for short FAs (e.g., octanoate) that preferably bind to Sudlow's site II (i.e., FA3-FA4) displacing the specific ligands (e.g., ibuprofen) without inducing HSA allosteric rearrangement(s) [10,12,13,17,20,51,52].

6. Conclusion and perspectives

Modulation of heme binding to HSA by drugs and metabolites is relevant in pharmacological therapy management. Indeed, the increase of the heme plasma level under pathological conditions (reaching ca. 5×10^{-5} M [39,79]), such as severe hemolytic anemia, crash syndrome, and post-ischemic reperfusion, could induce the release of HSA-bound endogenous and exogenous ligands (e.g., metabolites and drugs) increasing their bio-availability with the concomitant intoxication of the patient. As expected, the toxic plasma heme concentration could increase in patients after drug administration. Accounting for both the affinity constants of drug binding to HSA and HSA-heme-Fe, and the plasma levels of drugs binding to Sudlow's site I (and even to other functionally linked sites FA2 and FA6), the molar fraction of the drug-

bound HSA could decrease from 50–90% to 10–50% in the presence of specific ligands [9–11,13,16,80].

Drug-dependent $^{\bullet}$NO and peroxynitrite scavenging by HSA-heme-Fe could occur only in patients affected with a variety of severe hematologic diseases characterized by excessive intravascular hemolysis, displaying high HSA-heme-Fe plasmatic levels [39,79]. Although the in vivo concentration of $^{\bullet}$NO and peroxynitrite is openly debated, their levels have been reported to be much higher than micromolar concentration, at least over brief periods of time [81–87]. Finally, the plasma level of prototypical drugs ibuprofen and warfarin is ca. 10^{-6} M–10^{-4} M [88–91]. Remarkably, concentrations of HSA-heme-Fe, reactive nitrogen species, and drugs used for in vitro studies overlap levels observed in vivo [59,60,67].

Lastly, data available for HSA-heme describe a curious situation where heme binding to a non-classical heme-protein (i.e., HSA) confers (although transiently) ligand binding and (pseudo-)enzymatic properties. Note that the effects arising from heme binding to HSA might have some role in the regulation of biological functions. Since these actions are dependent on the transient interaction of a ligand (e.g., heme) with a carrier (e.g., HSA), they have been called "chronosteric" effects [56,92].

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