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Ibuprofen and warfarin modulate allosterically ferrous human serum heme–albumin nitrosylation

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ABSTRACT

Ferrous human serum heme–albumin (HSA–heme–Fe(II)) displays globin-like properties. Here, the effect of ibuprofen and warfarin on kinetics of HSA–heme–Fe(II) nitrosylation is reported. Values of the second-order rate constant for HSA–heme–Fe(II) nitrosylation (k_{on}) decrease from $6.3 \times 10^6\ M^{-1}\ s^{-1}$ in the absence of drugs, to $4.1 \times 10^5\ M^{-1}\ s^{-1}$ and $4.8 \times 10^5\ M^{-1}\ s^{-1}$, in the presence of saturating amounts of ibuprofen and warfarin, respectively, at pH 7.0 and 20.0 °C. From the dependence of k_{on} on the drug concentration, values of the dissociation equilibrium constant for ibuprofen and warfarin binding to HSA–heme–Fe(II) (i.e., $K = 3.2 \times 10^{-3}$ M and 2.6×10^{-4} M, respectively) were determined. The observed allosteric effects could indeed reflect ibuprofen and warfarin binding to the regulatory fatty acid binding site FA2, which brings about an alteration of heme coordination, slowing down HSA–heme–Fe(II) nitrosylation. Present data highlight the allosteric modulation of HSA–heme–Fe(II) reactivity by heterotropic effectors.

1. Introduction

Human serum albumin (HSA), the most abundant protein in plasma, is characterized by an extraordinary ligand binding capacity. Indeed, HSA provides a depot and carrier for many compounds, affects pharmacokinetics of many drugs, provides the metabolic modification of some ligands, renders potential toxins harmless by transporting them to disposal sites, accounts for most of the antioxidant capacity of the human serum, and displays (pseudo-) enzymatic properties [1–16].

The modular three-dimensional structure of HSA provides a variety of ligand binding sites (Fig. 1). Among others, HSA is able to bind up to nine equivalents of long-chain fatty acids (FAs) at multiple binding sites (labeled FA1 to FA9). FA1 has a strong affinity for the ferric heme (heme-Fe(III)), FA3 and FA4 together contribute to Sudlow's site II (i.e., to the ibuprofen primary site), and FA7 corresponds to Sudlow's site I (i.e., the warfarin binding cleft). Moreover, FA2 and FA6 are secondary ibuprofen sites. Remarkably, the heme pocket (i.e., the FA1 cleft) and sites FA2, FA3-FA4 (i.e., Sudlow's site II), FA6, and FA7 (i.e., Sudlow's site I) are allosterically-coupled, ibuprofen and warfarin acting as prototypical allosteric effectors [4,5,8–16,17–34].

Remarkably, HSA is crucial for heme scavenging, providing protection against free heme oxidative damage, limiting the access of pathogens to heme, and contributing to iron homeostasis by recycling the heme iron [10,15,35–37]. In turn, heme binding confers to HSA globin-like spectroscopic and reactivity properties [9,14,16,30,38]. In particular, heme–albumin (HSA–heme–Fe) has been reported to bind NO and to act as a NO and peroxynitrite scavenger, both HSA–heme ligand binding and detoxification properties are modulated allosterically by drugs [14,16,31,34,39–44].

Here, the effect of ibuprofen and warfarin on kinetics of ferrous HSA–heme (HSA–heme–Fe(II)) nitrosylation is reported. Ibuprofen and warfarin binding to FA2 impair allosterically HSA–heme–Fe(II) nitrosylation by inducing His146–Fe(II) coordination. Present data contribute to the description of the allosteric modulation of HSA–heme–Fe(II) reactivity by heterotropic effectors, highlighting the role of FA2 as the warfarin secondary binding cleft.

2. Materials and methods

HSA (essentially FA free), hemin (protoporphyrin IX-Fe(III)) chloride, ibuprofen, and warfarin were obtained from Sigma-Aldrich

Abbreviations: FA, fatty acid; heme-Fe(III), ferric heme; HSA, human serum albumin; HSA–heme–Fe, Heme–albumin; HSA–heme–Fe(II), ferrous HSA–heme–Fe; HSA–heme–Fe(II)-NO, ferrous nitrosylated HSA–heme–Fe.

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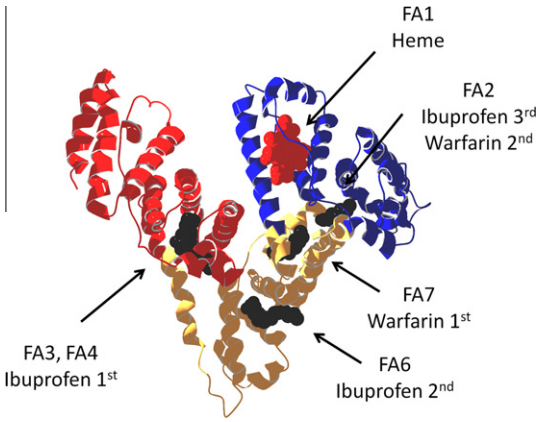

Fig. 1. Ribbon representation of the HSA structure highlighting the modular three-domain architecture (blue: domain I; orange: domain II; red: domain III). Relevant binding sites are labeled as follows: FA1 (heme site, occupied by heme, in red), FA2 (3rd ibuprofen site and 2nd warfarin site, occupied by myristate, in black), FA3 and FA4 (1st ibuprofen site, occupied by two myristates, in black), FA6 (2nd ibuprofen site, occupied by myristate, in black), and FA7 (1st warfarin site, occupied by myristate, in black). The coordinates of HSA complexed with heme and myristate were from PDB entry 1O9X [24]. The picture was drawn with Swiss-PDB-Viewer [52]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(St. Louis, MO, USA). NO (from Aldrich Chemical Co., Milwaukee, WI, USA) was purified by flowing it through an NaOH column in order to remove acidic nitrogen oxides. All the other chemicals (from Merck KGaA, Darmstadt, Germany) were of analytical or reagent grade and were used without purification unless stated.

HSA–heme–Fe(II) (2.2×10^{-6} M) was prepared by adding a 1.4-molar excess of HSA to the heme-Fe(II) solution (1.0×10^{-1} M sodium phosphate buffer, pH 7.0), at 20.0 °C [40,41,45].

The NO solution was prepared by keeping in a closed vessel the 1.0×10^{-1} M phosphate buffer solution (pH 7.0) under NO at P = 76.0 mm Hg anaerobically (T = 20.0 °C) [40,41].

The ibuprofen stock solution (2.0×10^{-2} M) was prepared by dissolving the drug in 1.0×10^{-1} M phosphate buffer, at pH 7.0 and 20.0 °C [41]. The warfarin stock solution (2.0×10^{-2} M) was prepared by dissolving the drug in water at pH 10.0, then adjusting pH to 7.0 with HCl [40]. Ibuprofen and warfarin stock solutions were then mixed with the HSA–heme–Fe(II) and NO solutions to obtain the desired final drug concentration.

The NO binding kinetics to HSA–heme–Fe(II) displays two phases, namely a fast ligand-dependent process (amounting to about 80% of the total absorption amplitude) and a slow ligand-independent process (amounting to about 20% of the total absorption amplitude) (see Fig. 1 of the Supplementary data). Kinetics of HSA–heme–Fe(II) nitrosylation were analysed in the framework of the minimum mechanism reported in Scheme 1, focussing only on the predominant fast bimolecular process.

The HSA–heme–Fe(II) nitrosylation process was regarded as irreversible because the rate of the inverse reaction is negligible with respect to values of the pseudo-first order rate constant for ferrous nitrosylated HSA–heme (HSA–heme–Fe(II)-NO) formation (k_{obs}) (see present study and [40,41] for comparison).

Values of k_{obs} for the fast ligand-dependent process were obtained by rapid-mixing the HSA–heme–Fe(II) (final concentration 1.1×10^{-6} M) solution with the NO (final concentration, 5.0×10^{-6}–3.0×10^{-5}) solution under anaerobic conditions, at pH 7.0 (1.0×10^{-1} M sodium phosphate buffer) and 20.0 °C, in the absence and presence of ibuprofen and warfarin (final concentration, 5.0×10^{-5}–1.0×10^{-2} M). Kinetics was monitored between 340 and 460 nm. The dead-time of the rapid-mixing stopped-flow apparatus was 1.4 ms.

$$\text{HSA-heme-Fe(II)} + \text{NO} \xrightarrow{k_{on}} \text{HSA-heme-Fe(II)-NO}$$

Scheme 1.

Values of k_{obs} for the fast ligand-dependent process were determined from data analysis according to Eq. (1):

$$[\text{HSA—heme—Fe(II)}]_t = [\text{HSA—heme—Fe(II)}]_i \times e^{-k_{obs} \times t} \quad (1)$$

Values of the second-order rate constants for NO binding to HSA–heme–Fe(II) (k_{on}; see Scheme 1) were obtained, in the absence and presence of ibuprofen and warfarin, from the dependence of k_{obs} on the NO concentration (i.e., [NO]) according to Eq. (2):

$$k_{obs} = k_{on} \times [\text{NO}] \quad (2)$$

Values of the dissociation equilibrium constant for ibuprofen and warfarin binding to HSA–heme–Fe(II) (i.e., K) were obtained from the dependence of k_{on} on the drug concentration (i.e., [drug]). Values of K were determined from data analysis, according to Eq. (3):

$$k_{on} = (k^*_{on} - k^0_{on}) - \{((k^*_{on} - k^0_{on}) \times [\text{drug}])/(K + [\text{drug}])\} + k^0_{on} \quad (3)$$

where k^*_{on} is the k_{on} value obtained in the absence of drugs (i.e., under conditions where [drug] = 0), and k^0_{on} is the k_{on} value obtained in the presence of saturating amounts of drugs (i.e., under conditions where [drug] >> K).

Kinetic data were analyzed using the MatLab program (The Math Works Inc., Natick, MA, USA). The results are given as mean values of at least four experiments plus or minus the corresponding standard deviation.

3. Results and discussion

In the absence of drugs, mixing of the HSA–heme–Fe(II) and NO solutions causes a shift of the optical absorption maximum of the Soret band (i.e., λ_{max}) from 418 nm (i.e., HSA–heme–Fe(II)) to 389 nm (i.e., HSA–heme–Fe(II)–NO). In the presence of saturating amounts of ibuprofen and warfarin (1.0×10^{-2} M), mixing of the HSA–heme–Fe(II) and NO solutions causes a shift of λ_{max} from 422 nm (*i.e.*, drug-HSA–heme–Fe(II)) to 418 nm (i.e., drug-HSA–heme–Fe(II)–NO). These spectroscopic observations agree with previous data [19,20,31,46] indicating that the heme–Fe atom of drug-free HSA–heme–Fe(II) and of HSA–heme–Fe(II)–NO is predominantly tetra- and penta-coordinated, respectively, whereas the heme–Fe atom of drug-bound HSA–heme–Fe(II) and of HSA–heme–Fe(II)-NO is predominantly penta- and hexa-coordinated, respectively.

Under all the experimental conditions, the time course for NO binding to HSA–heme–Fe(II) is biphasic (see Fig. 1 of the Supplementary Data). The fast and the slow processes account for about 80% and 20% of the total absorption amplitude, respectively. Noticeably, the fast process displays a typical bimolecular (NO-dependent) behavior and is affected by drug concentration. On the other hand, the slow process is independent of the NO and drug concentration. In this context, data were analysed in the framework of the minimum mechanism reported in Scheme 1, focussing only on the predominant fast bimolecular process.

The time course of the fast bimolecular nitrosylation process of HSA–heme–Fe(II), in the absence and presence of ibuprofen and warfarin, is shown in Figs. 2 and 3 (panel A). Values of the pseudo-first-order rate constant for fast bimolecular nitrosylation of HSA–heme–Fe(II) (i.e., k_{obs}; see Eq. (1)) are wavelength-independent at fixed NO and drug concentration (data not shown).

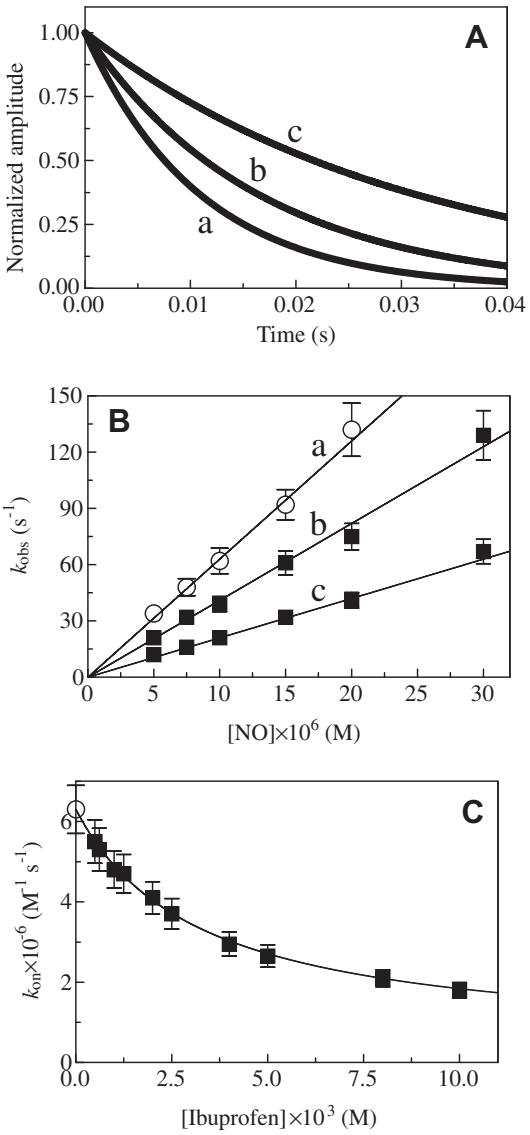

Fig. 2. Effect of ibuprofen on the HSA–heme–Fe(II) nitrosylation. Normalized averaged time courses of NO binding to HSA–heme–Fe(II) in the absence and presence of ibuprofen (A). The ibuprofen concentration was 0.0 M (trace a), 2.0×10^{-3} M (trace b), and 8.0×10^{-3} M (trace c). The time course analysis according to Eq. (1) allowed the determination of the following values of k_{obs}: $9.2 \times 10\ s^{-1}$ (trace a), $6.1 \times 10\ s^{-1}$ (trace b), and $3.2 \times 10\ s^{-1}$ (trace c). The HSA–heme–Fe(II) concentration was 1.1×10^{-6} M. The NO concentration was 1.5×10^{-5} M. Dependence of k_{obs} on the NO concentration in the absence and presence of ibuprofen (B). The ibuprofen concentration was: 0.0 M (a), 2.0×10^{-3} M (b), and 8.0×10^{-3} M (c). The continuous lines were calculated according to Eq. (2) with the following values of $k_{on} = 6.3 \times 10^6\ M^{-1}\ s^{-1}$ (a), $4.1 \times 10^6\ M^{-1}\ s^{-1}$ (b), and $2.1 \times 10^6\ M^{-1}\ s^{-1}$ (c). Dependence of k_{on} on the ibuprofen concentration (C). The continuous line was calculated according to Eq. (3) with the following parameters: $k^*_{on} = 6.3 \times 10^6\ M^{-1}\ s^{-1}$, $k^0_{on} = 4.1 \times 10^5\ M^{-1}\ s^{-1}$, and $K = 3.2 \times 10^{-3}$ M. All data were obtained at pH 7.0 and 20.0 °C. Where not shown, standard deviation is smaller than the symbol. For details, see text.

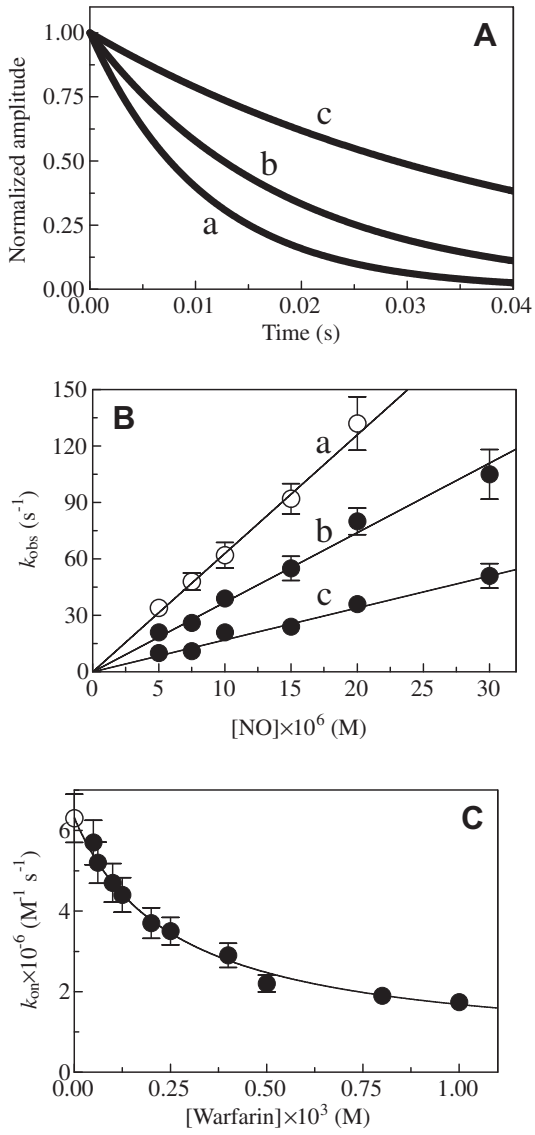

Fig. 3. Effect of warfarin on the HSA–heme–Fe(II) nitrosylation. Normalized averaged time courses of NO binding to HSA–heme–Fe(II) in the absence and presence of warfarin (A). The warfarin concentration was 0.0 M (trace a), 2.0×10^{-4} M (trace b), and 1.0×10^{-3} M (trace c). The time course analysis according to Eq. (1) allowed the determination of the following values of k_{obs}: $9.2 \times 10^1\ s^{-1}$ (trace a), $5.5 \times 10^1\ s^{-1}$ (trace b), and $2.4 \times 10^1\ s^{-1}$ (trace c). The HSA–heme–Fe(II) concentration was 1.1×10^{-6} M. The NO concentration was 1.5×10^{-5} M. Dependence of k_{obs} on the NO concentration in the absence and presence of warfarin (B). The warfarin concentration was: 0.0 M (a), 2.0×10^{-4} M (b), and 1.0×10^{-3} M (c). The continuous lines were calculated according to Eq. (2) with the following values of $k_{on} = 6.3 \times 10^6\ M^{-1}\ s^{-1}$ (a), $3.7 \times 10^6\ M^{-1}\ s^{-1}$ (b), and $1.7 \times 10^6\ M^{-1}\ s^{-1}$ (c). Dependence of k_{on} on the warfarin concentration (C). The continuous line was calculated according to Eq. (3) with the following parameters: $k^*_{on} = 6.3 \times 10^6\ M^{-1}\ s^{-1}$, $k^0_{on} = 4.8 \times 10^5\ M^{-1}\ s^{-1}$, and $K = 2.6 \times 10^{-4}$ M. All data were obtained at pH 7.0 and 20.0 °C. Where not shown, standard deviation is smaller than the symbol. For details, see text.

Values of k_{obs} increase linearly with the NO concentration, in the absence and presence of ibuprofen and warfarin (Figs. 2 and 3, panel B). The analysis of data reported in Figs. 2 and 3 (panel B) according to Eq. (2) allowed the determination of values of the second-order rate constant for HSA–heme–Fe(II) nitrosylation (k_{on}; corresponding to the slope of the linear plots). Remarkably, the *y* intercept of the linear plots (corresponding to the first-order rate constant for NO dissociation from HSA–heme–Fe(II)–NO) is very close to zero, indicating that HSA–heme–Fe(II) nitrosylation can be considered as an irreversible process over the time range explored. Indeed, values of the first-order rate constant for NO dissociation from (drug-)HSA–heme–Fe(II)–NO [40,41] are lower than those of k_{obs} (see Figs. 2 and 3, panels B and C) by 4–6 orders of magnitude.

Values of k_{on} for HSA–heme–Fe(II) nitrosylation decrease from $6.3 \times 10^6\ M^{-1}\ s^{-1}$ in the absence of drugs, to $4.1 \times 10^5\ M^{-1}\ s^{-1}$ and $4.8 \times 10^5\ M^{-1}\ s^{-1}$, in the presence of saturating amounts of ibuprofen and warfarin, respectively. The k^*_{on} value here reported ($= 6.3 \times 10^6\ M^{-1}\ s^{-1}$) is similar to that obtained for NO binding to HSA-tetraphenylporphynatoiron(II) ($= 1.5 \times 10^7\ M^{-1}\ s^{-1}$) [47]. The

analysis of the dependence of k_{on} on the drug concentration (Figs. 2 and 3, panel C), according to Eq. (3), allowed to determine values of the dissociation equilibrium constant for ibuprofen and warfarin binding to HSA–heme–Fe(II) (i.e., $K = 3.2 \times 10^{-3}$ and 2.6×10^{-4} M, respectively).

However, any energy balancing analysis of thermodynamic and kinetic parameters for HSA–heme–Fe(II)(–NO) (de)nitrosylation, in the absence and in the presence of ibuprofen and warfarin, is impaired by the evidence that the coordination state of HSA–heme–Fe(II)(–NO) is altered to a relevant extent upon drug binding. In fact, in the absence of allosteric effectors, HSA–heme–Fe(II)-NO is predominantly five-coordinated. In contrast, HSA–heme–Fe(II)-NO becomes predominantly hexa-coordinated upon addition of ibuprofen and warfarin [19,20,31].

Data reported in Figs. 2 and 3 (panel C) indicate that ibuprofen and warfarin bind to one site of HSA–heme–Fe(II). Warfarin binds to one site of HSA–heme–Fe(II)-NO ($K = 6.2 \times 10^{-5}$ M) [40], of HSA–heme–Fe(III) ($K = 2.1 \times 10^{-5}$ M) [19], and of heme-free HSA ($K = 3.0 \times 10^{-6}$ M) [19] also. In contrast, ibuprofen binds to three independent sites of HSA–heme–Fe(II)–NO ($K_1 = 3.1 \times 10^{-7}$ M, $K_2 = 1.7 \times 10^{-4}$ M, and $K_3 = 2.2 \times 10^{-3}$ M) [41] and of HSA–heme–Fe(III) ($K_1 = 8.0 \times 10^{-8}$ M, $K_2 = 5.0 \times 10^{-5}$ M, and $K_3 = 7.7 \times 10^{-4}$ M) [42]. Moreover, ibuprofen binds to two sites of heme-free HSA ($K_1 = 3.7 \times 10^{-7}$ M and $K_2 = 4.0 \times 10^{-5}$ M) [2,33,48]. These findings indicate that ibuprofen and warfarin bind to several HSA–heme–Fe sites with different affinity. Moreover, the drug affinity for HSA–heme–Fe depends on the redox state and the (un)ligated form of the heme–Fe atom.

The ibuprofen primary binding cleft (i.e., Sudlow's site II) is formed by the FA3 and FA4 sites, the secondary binding pocket corresponds to FA6 [11], and the third low affinity cleft has been identified with the FA2 pocket [26,30,31,34,42]. The warfarin primary binding site (i.e., Sudlow's site I) corresponds to FA7 [11]. A secondary low-affinity warfarin binding pocket has been postulated on the basis of binding experiments to recombinant HSA fragments [49], but not characterized in full-length HSA from both structural and functional viewpoints. By taking into account values of the dissociation equilibrium constants for ibuprofen and warfarin binding to HSA–heme–Fe(II) observed here (i.e., $K = 3.2 \times 10^{-3}$ and 2.6×10^{-4} M, respectively), it is feasible to consider the drug low-affinity site(s) as responsible for the observed effect [31,33,34,41,42].

Although the low-affinity sites for ibuprofen and warfarin binding to HSA were never described by X-ray crystallography, there are evidences from solution studies that ibuprofen binds to three different sites, the weakest one corresponding to FA2 [31,34]. In a similar way, warfarin binds to two different sites, the lowest affinity one being located at the interface between domains IA and II [49], thus corresponding to FA2. Drug binding to FA2 has been postulated to induce a remarkable conformational change(s) in HSA with the consequent reorientation of His146 that was suggested as the putative ligand able to coordinate to the heme iron at the sixth position [31,34]. Remarkably, His146 has been demonstrated to be indispensable for the allosteric transition(s) of HSA upon ligand binding to FA2 [50].

The drug-dependent decrease of k_{on} for NO binding to HSA–heme–Fe(II) (present study) could reflect the tetra- to penta-coordination transition of the heme-Fe(II) atom. Spectroscopic observations here reported, i.e. the large red shift of the Soret band upon drug binding, suggest the drug-dependent His146–Fe(II) coordination, which inhibits HSA–heme–Fe(II) nitrosylation. A similar behavior has been reported for modulation of CO binding to oxygen carriers, such as myoglobin [51]. Remarkably, drugs impair peroxynitrite scavenging by HSA–heme–Fe(III) by inducing the formation of the six-coordinated His146-Fe(III)-Tyr161 species, rendering unreactive the heme–Fe atom [31,42,43].

As a whole, present data reinforce the idea that HSA could be taken as the prototype of monomeric allosteric proteins. Furthermore, HSA–heme represents a unique case within heme–proteins since allosteric effectors modulate both heme binding to HSA and the heme–Fe atom reactivity [14,16].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.130.

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