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O_2-mediated oxidation of ferrous nitrosylated human serum heme–albumin is limited by nitrogen monoxide dissociation

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ABSTRACT

Human serum heme–albumin (HSA-heme-Fe) displays globin-like properties. Here, kinetics of O_2-mediated oxidation of ferrous nitrosylated HSA-heme-Fe (HSA-heme-Fe(II)-NO) is reported. Values of the first-order rate constants for O_2-mediated oxidation of HSA-heme-Fe(II)-NO (*i.e.*, for ferric HSA-heme-Fe formation) and for NO dissociation from HSA-heme-Fe(II)-NO (*i.e.*, for NO replacement by CO) are $k = 9.8 \times 10^{-5}$ and 8.3×10^{-4} s^{-1}, and $h = 1.3 \times 10^{-4}$ and 8.5×10^{-4} s^{-1}, in the absence and presence of rifampicin, respectively, at pH = 7.0 and $T = 20.0$ °C. The coincidence of values of k and h indicates that NO dissociation represents the rate limiting step of O_2-mediated oxidation of HSA-heme-Fe(II)-NO. Mixing HSA-heme-Fe(II)-NO with O_2 does not lead to the formation of the transient adduct(s), but leads to the final ferric HSA-heme-Fe derivative. These results reflect the fast O_2-mediated oxidation of ferrous HSA-heme-Fe and highlight the role of drugs in modulating allosterically the heme-Fe-atom reactivity.

1. Introduction

Human serum albumin (HSA), the most abundant protein in plasma (reaching a blood concentration of about 7.0×10^{-4} M), participates to heme scavenging, providing protection against free heme oxidative damage, limiting access by pathogens to heme, and contributing to iron homeostasis by recycling the heme iron (see [1–4]). In turn, heme-Fe endows HSA with reactivity and spectroscopic properties similar to those of heme-globins. Remarkably, both ferric heme-Fe (heme-Fe(III)) binding to HSA and human serum heme–albumin (HSA-heme-Fe) reactivity are modulated allosterically [1,5–9].

As reported for some globins [10–18], ferric HSA-heme-Fe (HSA-heme-Fe(III) and ferrous nitrosylated HSA-heme-Fe (HSA-heme-Fe(II)-NO) facilitate peroxynitrite scavenging [8,19–22]. However, the fast O_2-mediated oxidation of ferrous HSA-heme-Fe (HSA-heme-Fe(II)) [23] prevents NO detoxification by ferrous oxygenated HSA-heme-Fe (HSA-heme-Fe(II)-O_2).

Here, the effect of isoniazid, an anti-tuberculosis drug binding to Sudlow's site I [22], on the reaction of HSA-heme-Fe(II)-NO with O_2 is reported. In contrast with some globins [12,24–26], mixing HSA-heme-Fe(II)-NO with O_2 does not lead to the formation of the transient HSA-heme-Fe(III)-peroxynitrite adduct, but to the final HSA-heme-Fe(III) derivative. In agreement with the fast O_2-mediated oxidation of HSA-heme-Fe(II) [23], the rate limiting step of the O_2-mediated oxidation of HSA-heme-Fe(II)-NO is represented by NO dissociation.

2. Materials

HSA (96%, essentially fatty acid free), hemin (Fe(III)-protoporphyrin IX) chloride, and rifampicin were obtained from Sigma–Aldrich (St. Louis, MO, USA). CO was purchased from Linde AG (Höllriegelskreuth, Germany). NO (from Aldrich Chemical Co., Milwaukee, WI, USA) was purified by flowing through an NaOH column in order to remove acidic nitrogen oxides. O_2 was obtained

Abbreviations: heme-Fe(III), ferric heme-Fe; HSA, human serum albumin; HSA-heme-Fe, human serum heme–albumin; HSA-heme-Fe(II), ferrous HSA-heme-Fe; HSA-heme-Fe(II)-CO, ferrous carbonylated HSA-heme-Fe; HSA-heme-Fe(II)-O_2, ferrous oxygenated HSA-heme-Fe; HSA-heme-Fe(II)-NO, ferrous nitrosylated HSA-heme-Fe; HSA-heme-Fe(III), ferric HSA-heme-Fe; Hb(II)-NO, ferrous nitrosylated hemoglobin; HPX-heme-Fe(II)-NO, ferrous nitrosylated hemopexin-heme; HPX-heme-Fe(III), ferric hemopexin-heme; HPX-heme-Fe(III)-N(O)OO, peroxynitrite-bound ferric hemopexin-heme; Mb(II)-NO, ferrous nitrosylated myoglobin; Mb(III), ferric myoglobin; Mb(III)-OONO, peroxynitrite-bound ferric myoglobin; Ngb(II)-NO, ferrous nitrosylated neuroglobin; Ngb(III), ferric neuroglobin.

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from Linde Caracciolossigeno SRL (Roma, Italy). All products were of analytical or reagent grade and were used without further purification unless stated.

HSA-heme-Fe(III) (=3.2×10^{-6} M) was prepared by adding a 1.4-M excess of HSA solution (1.0×10^{-1} M sodium phosphate buffer, pH = 7.0) to the heme-Fe(III) solution (1.0×10^{-2} M NaOH) at T = 20.0 °C. HSA-heme(II)-NO (=3.2×10^{-6} M) was obtained, under anaerobic conditions, by blowing purified NO over the HSA-heme-Fe(III) solution (1.0×10^{-1} M sodium phosphate buffer, pH = 7.0) at T = 20.0 °C [22]. The excess of NO was gently pumped off before each kinetic experiment.

The CO, NO, and O_2 stock solutions were prepared by keeping in a closed vessel the 1.0×10^{-1} M sodium phosphate buffer solution (pH = 7.0) under CO or NO or O_2 at P = 760.0 mm Hg and T = 20.0 °C. The solubility of CO, NO, and O_2 in the aqueous buffered solution is 1.03×10^{-3}, 2.05×10^{-3}, and 1.38×10^{-3} M, respectively, at P = 760.0 mm Hg and T = 20.0 °C [27]. All the other chemicals were obtained from Merck AG (Darmstadt, Germany). The rifampicin stock solution (=2.0×10^{-2} M) was prepared by dissolving the drug in methanol [22].

3. Methods

Values of the first-order rate constant for O_2-mediated conversion of HSA-heme-Fe(II)-NO to HSA-heme-Fe(III) (*i.e.*, k) were determined spectrophotometrically by mixing the HSA-heme-Fe(II)-NO solution (final concentration, 1.6×10^{-6} M) with the O_2 solution (final concentration, 1.0×10^{-4}–5.0×10^{-4} M) in the absence and presence of rifampicin (final concentration, 5.0×10^{-5} and 1.0×10^{-2} M), at pH = 7.0 (1.0×10^{-1} M sodium phosphate buffer) and T = 20.0 °C. No gaseous phase was present. Kinetics was monitored between 350 and 460 nm. The time courses were fitted to a mono-exponential process according to the minimum reaction mechanism depicted in Scheme 1.

Values of k have been determined from data analysis, according to Eq. (1):

$$[\text{HSA} - \text{heme} - \text{Fe(II)} - \text{NO}]_t = [\text{HSA} - \text{heme} - \text{Fe(II)} - \text{NO}]_i \times e^{-k \times t} \quad (1)$$

Values of the first-order rate constant for NO dissociation from HSA-heme-Fe(II)-NO (*i.e.*, for NO replacement by CO; h) were determined by mixing the HSA-heme-Fe(II)-NO solution (final concentration, 3.2×10^{-6} M) with the CO-dithionite solution (final concentration, 1.0×10^{-4}–5.0×10^{-4} M, and 2.0×10^{-5} M, respectively) in the absence and presence of rifampicin (final concentration, 5.0×10^{-5} and 1.0×10^{-2} M), at pH = 7.0 (1.0×10^{-1} M sodium phosphate buffer), and T = 20.0 °C. No gaseous phase was present [28]. Kinetics was monitored between 350 and 460 nm. The time course of NO dissociation from HSA-heme-Fe(II)-NO (*i.e.*, of NO replacement by CO) was fitted to a single exponential process according to the minimum reaction mechanism represented by Scheme 2 [28].

Values of h have been determined from data analysis, according to Eq. (2) [28]:

$$[\text{HSA} - \text{heme} - \text{Fe(II)} - \text{NO}]_t = [\text{HSA} - \text{heme} - \text{Fe(II)} - \text{NO}]_i \times e^{-h \times t} \quad (2)$$

Values of the dissociation equilibrium constant for rifampicin binding to HSA-heme-Fe(II)-NO (L) were determined from the dependence of k and h values on the drug concentration (ranging between 5.0×10^{-5} and 1.0×10^{-2} M), according to Eq. (3) [8,22]:

$$b = (b_{top} \times [\text{rifampicin}])/(L + [\text{rifampicin}]) + b_0 \quad (3)$$

where b_{top} represents the asymptotic value of b under conditions where [rifampicin] >> L, and b_0 indicates the value of b under conditions where [rifampicin] = 0; b is either k or h, b_{top} is either k_{top} or h_{top}, and b_0 is either k_0 or h_0.

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

4. Results

4.1. Kinetics of O_2-mediated oxidation of HSA-heme-Fe(II)-NO

In the absence of rifampicin, mixing HSA-heme-Fe(II)-NO and O_2 solutions (at pH = 7.0 and T = 20.0 °C) causes a shift of the optical absorption maximum of the Soret band from 389 nm (*i.e.*, HSA-heme-Fe(II)-NO) to 403 nm (*i.e.*, HSA-heme-Fe(III)) and a change of the extinction coefficient from $\varepsilon_{389\ nm} = 6.4 \times 10^4\ M^{-1}\ cm^{-1}$ (*i.e.*, HSA-heme-Fe(II)-NO) to $\varepsilon_{403\ nm} = 1.1 \times 10^5\ M^{-1}\ cm^{-1}$ (*i.e.*, HSA-heme-Fe(III)).

In the presence of rifampicin ($\geqslant 5.0 \times 10^{-3}$ M), mixing HSA-heme-Fe(II)-NO and O_2 solutions (at pH = 7.0 and T = 20.0 °C) causes a shift of the optical absorption maximum of the Soret band from 418 nm (*i.e.*, HSA-heme-Fe(II)-NO) to 375 nm (*i.e.*, HSA-heme-Fe(III)) and a change of the extinction coefficient from $\varepsilon_{418\ nm} = 1.3 \times 10^5\ M^{-1}\ cm^{-1}$ (*i.e.*, HSA-heme-Fe(II)-NO) to $\varepsilon_{375\ nm} = 8.2 \times 10^4\ M^{-1}\ cm^{-1}$ (*i.e.*, HSA-heme-Fe(III)).

As reported for ligands binding to Sudlow's site I [7,19], differences in the absorption spectra of HSA-heme-Fe(II)-NO and HSA-heme-Fe(III) obtained in the absence and presence of rifampicin reflect drug binding to HSA-heme-Fe derivatives [22]. In the absence and presence of rifampicin, the optical absorption spectra of HSA-heme-Fe(II)-NO and HSA-heme-Fe(III), obtained by mixing the HSA-heme-Fe(II)-NO and O_2 solutions, correspond to those reported in the literature [19,22]. In this respect, it is important to outline that absorption spectra of HSA-heme-Fe(II)-NO in the absence of rifampicin correspond to a penta-coordinate heme form,

$$\text{HSA-heme-Fe(II)-NO} + O_2 \xrightarrow{k} \text{HSA-heme-Fe(II)} + O_2 + \text{NO} \xrightarrow{fast} \rightarrow \text{HSA-heme-Fe(III)} + O_2^{\bullet -}$$

Scheme 1.

$$\text{HSA-heme-Fe(II)-NO} + \text{CO} \xrightarrow{h} \text{HSA-heme-Fe(II)} + \text{NO} + \text{CO} \xrightarrow{fast} \text{HSA-heme-Fe(II)-CO} + \text{NO}$$

Scheme 2.

where the proximal axial ligand has been cleaved off, as also observed by EPR spectroscopy [29–31]; on the other hand, the addition of rifampicin restores the hexa-coordination of the heme, as observed also for other heterotropic ligands of HSA-heme-Fe(II)-NO [29–31]. Moreover, the variations of the optical absorption spectra of HSA-heme-Fe(II)-NO and HSA-heme-Fe(III) are independent of the type of ligands binding to Sudlow's site I [7,19,29].

Over the whole O_2 concentration range explored, the time course for the O_2-mediated oxidation of HSA-heme-Fe(II)-NO corresponds to a [O_2]-independent mono-molecular process for more than 90% of its course, in the absence and presence of rifampicin (Fig. 1A). Moreover, values of the first-order rate constant for O_2-mediated oxidation of HSA-heme-Fe(II)-NO (*i.e.*, for HSA-heme-Fe(III) formation; k) are wavelength-independent (data not shown). In the absence of rifampicin, the average value of k is 9.8×10^{-5} s^{-1} (Fig. 1B). In contrast, values of k increase with the rifampicin concentration, tending to level off at [drug] $\geqslant 5.0 \times 10^{-3}$ M (Fig. 1C and D). The analysis of data shown in Fig. 1D according to Eq. (3) allowed to calculate values of L (=4.4×10^{-4} M) and k_{top} (=8.3×10^{-4} s^{-1}).

4.2. Kinetics of NO dissociation from HSA-heme-Fe(II)-NO

Mixing HSA-heme-Fe(II)-NO and CO solutions (at pH = 7.0 and T = 20.0 °C) causes a shift of the optical absorption maximum of the Soret band from 389 nm (*i.e.*, HSA-heme-Fe(II)-NO) to 415 nm (*i.e.*, ferrous carbonylated HSA-heme-Fe; HSA-heme-Fe(II)-CO) and a change of the extinction coefficient from $\varepsilon_{389\ nm} = 6.4 \times 10^4$ M^{-1} cm^{-1} (*i.e.*, HSA-heme-Fe(II)-NO) to $\varepsilon_{415\ nm} = 1.2 \times 10^5$ M^{-1} cm^{-1} (*i.e.*, HSA-heme-Fe(II)-CO).

In the presence of rifampicin ($\geqslant 5.0 \times 10^{-3}$ M), mixing HSA-heme-Fe(II)-NO and CO solutions (at pH = 7.0 and T = 20.0 °C) causes a shift of the optical absorption maximum of the Soret band from 418 nm (*i.e.*, HSA-heme-Fe(II)-NO) to 414 nm (*i.e.*, HSA-heme-Fe(II)-CO) and a change of the extinction coefficient from $\varepsilon_{418\ nm} = 1.3 \times 10^5$ M^{-1} cm^{-1} (*i.e.*, HSA-heme-Fe(II)-NO) to $\varepsilon_{414\ nm} = 1.6 \times 10^5$ M^{-1} cm^{-1} (*i.e.*, HSA-heme-Fe(II)-CO).

Differences in the absorption spectra of HSA-heme-Fe(II)-NO and HSA-heme-Fe(II)-CO obtained in the absence and presence of rifampicin reflect drug binding to HSA-heme derivatives. In the absence and presence of rifampicin, the optical absorption spectra of HSA-heme-Fe(II)-NO and HSA-heme-Fe(II)-CO, obtained by mixing the HSA-heme-Fe(II)-NO and CO solutions, correspond to those reported in the literature [7,19,32].

Over the whole CO concentration range explored, the time course of HSA-heme-Fe(II)-NO denitrosylation (*i.e.*, for NO replacement by CO) corresponds to a [CO]-independent mono-molecular process for more than 90% of its course (Fig. 2A). Moreover, values of the first-order rate constant for HSA-heme-Fe(II)-NO denitrosylation (*i.e.*, for HSA-heme-Fe(II)-CO formation; h) are wavelength-independent (data not shown). In the absence of rifampicin, the average value of h is 1.3×10^{-4} s^{-1} (Fig. 2B). In contrast, values of h increase with the rifampicin concentration, tending to level off at [drug] $\geqslant 5.0 \times 10^{-3}$ M (Fig. 2C and D). The analysis of data shown in Fig. 2D according to Eq. (3) allowed to calculate values of L (=4.6×10^{-4} M) and ($h_{top} = 8.8 \times 10^{-4}$ s^{-1}). The h_0 value here obtained agrees with those reported previously [7,8,28]. Moreover, the h_{top} value here obtained at [rifampicin] $\geqslant 5.0 \times 10^{-3}$ M is closely similar to those obtained in the presence of saturating levels of abacavir and warfarin [7]. This indicates that the increase of h upon drug binding to Sudlow's site I is a ligand-independent event.

5. Discussion

Data here reported represent the first evidence for O_2-mediated oxidation of HSA-heme-Fe(II)-NO. Under all the experimental conditions, mixing HSA-heme-Fe(II)-NO with O_2 does not lead to

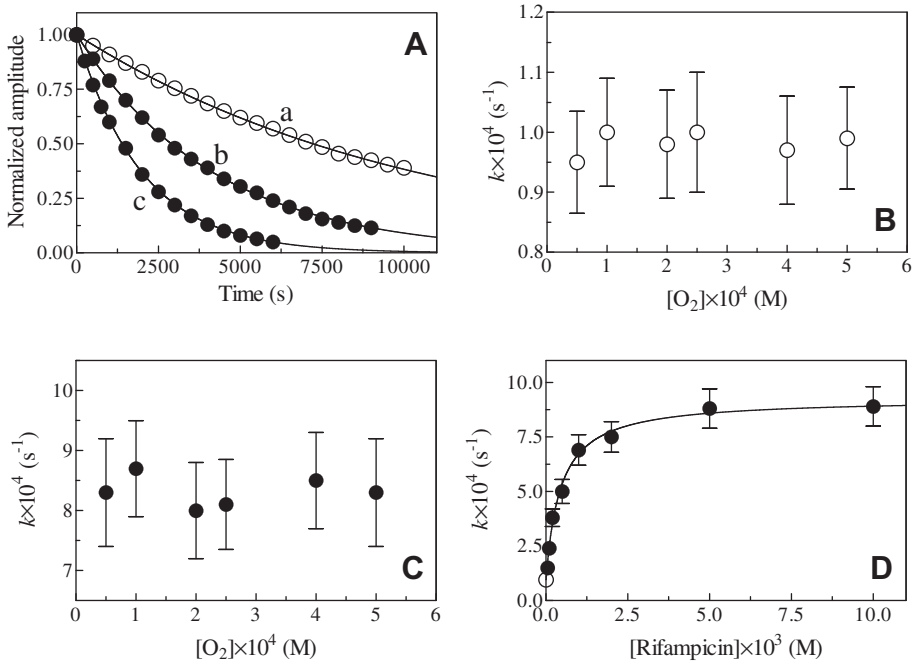

Fig. 1. O_2-mediated oxidation of HSA-heme-Fe(II)-NO, at pH = 7.0 and T = 20.0 °C. (A) Normalized averaged time courses of O_2-mediated oxidation of HSA-heme-Fe(II)-NO in the absence and presence of rifampicin. The rifampicin concentration was 0.0 M (trace a), 1.0×10^{-4} M (trace b), and 5.0×10^{-4} M (trace c). The time course analysis according to Eq. (1) allowed the determination of the following values of k: trace a, $k = 9.6 \times 10^{-5}$ s^{-1}; trace b, $k = 2.4 \times 10^{-4}$ s^{-1}; and trace c, $k = 5.0 \times 10^{-4}$ s^{-1}. (B) Dependence of k on the O_2 concentration in the absence of rifampicin. The average value of k is $(9.8 \pm 0.2) \times 10^{-5}$ s^{-1}. (C) Dependence of k on the O_2 concentration in the presence of rifampicin. The rifampicin concentration was 5.0×10^{-3} M. The average value of k is $(8.3 \pm 0.9) \times 10^{-4}$ s^{-1}. (D) Dependence of k on the rifampicin concentration. The open circle on the ordinate indicates the k value obtained in the absence of rifampicin (corresponding to k_0). The continuous line was calculated according to Eq. (3) with $L = (4.4 \pm 0.5) \times 10^{-4}$ M, $k_{top} = (8.3 \pm 0.8) \times 10^{-4}$ s^{-1}, and $k_0 = (9.6 \pm 1.0) \times 10^{-5}$ s^{-1}. Where not shown, standard deviation is smaller than the symbol. For details, see text.

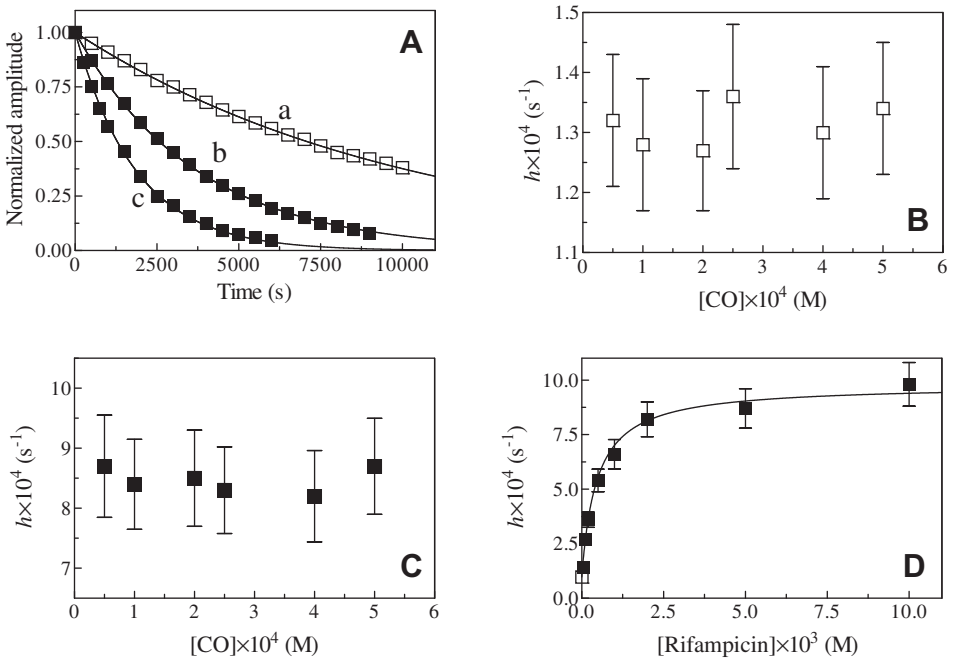

Fig. 2. NO dissociation from HSA-heme-Fe(II)-NO, at pH = 7.0 and T = 20.0 °C. (A) Normalized averaged time courses of NO dissociation from HSA-heme-Fe(II)-NO in the absence and presence of rifampicin. The rifampicin concentration was 0.0 M (trace a), 1.0×10^{-4} M (trace b), and 5.0×10^{-4} M (trace c). The time course analysis according to Eq. (1) allowed the determination of the following values of h: trace a, $h = 9.8 \times 10^{-5}$ s^{-1}; trace b, $h = 2.7 \times 10^{-4}$ s^{-1}; and trace c, $h = 5.4 \times 10^{-4}$ s^{-1}. (B) Dependence of h on the CO concentration in the absence of rifampicin. The average value of h is $(1.3 \pm 0.2) \times 10^{-4}$ s^{-1}. (C) Dependence of h on the CO concentration in the presence of rifampicin. The rifampicin concentration was 5.0×10^{-3} M. The average value of h is $(8.5 \pm 0.8) \times 10^{-4}$ s^{-1}. (D) Dependence of h on the rifampicin concentration. The open square on the ordinate indicates the h value obtained in the absence of rifampicin (corresponding to h_0). The continuous line was calculated according to Eq. (3) with $L = (4.6 \pm 0.5) \times 10^{-4}$ M, $h_{top} = (8.8 \pm 0.9) \times 10^{-4}$ s^{-1}, and $h_0 = (9.8 \pm 0.9) \times 10^{-5}$ s^{-1}. Where not shown, standard deviation is smaller than the symbol. For details, see text.

the formation of the transient adducts, which have been observed in the O_2-mediated oxidation of some heme-proteins [12,16,24,33,34], but leads to the final HSA-heme-Fe(III) derivative. The close similarity (within the error limits) for values of kinetic parameters k and h (see Figs. 1 and 2) indicates that NO dissociation represents the rate limiting step of O_2-mediated oxidation of HSA-heme-Fe(II)-NO. This clearly indicates that HSA-heme-Fe(II), formed by NO dissociation (see Scheme 1), is oxidized to HSA-heme-Fe(III) at a much faster rate, rendering undetectable any intermediate species (see Scheme 1), according to literature [23].

This behavior is drastically different from that reported for O_2-mediated oxidation of ferrous nitrosylated globins. Thus, in the case of ferrous nitrosylated horse heart myoglobin (Mb(II)-NO), the rate limiting step for the O_2-mediated oxidation is represented by the dissociation of the transient peroxynitrite-bound ferric myoglobin (Mb(III)-OONO). This reaction appears to be characterized by: (i) NO dissociation, (ii) heme dioxygenation, (iii) formation of the Mb(III)-OONO species, (iv) peroxynitrite isomerization, and (v) formation of ferric myoglobin (Mb(III)) and nitrate [24,33,34]. However, in the case of ferrous nitrosylated human hemoglobin (Hb(II)-NO), though following the same reaction mechanism reported for the O_2-mediated oxidation of Mb(II)-NO, NO dissociation has been reported to be the rate limiting step for the O_2-mediated oxidation, wherefore inositol hexakisphosphate affects this reaction by shifting the quaternary equilibrium from the R to the T state [25]. In contrast, O_2 reacts directly with ferrous nitrosylated rabbit hemopexin-heme (HPX-heme-Fe(II)-NO), this leads to the formation of the transient peroxynitrite-bound ferric hemopexin-heme species (HPX-heme-Fe(III)-N(O)OO), which precedes the appearance of the final products, *i.e.* ferric hemopexin-heme (HPX-heme(III)) and nitrate [19]. Lastly, a slight rearrangement within the protein structure, which may take place after formation of ferric human neuroglobin (Ngb(III)), has been postulated to be the rate limiting step in O_2-mediated oxidation of ferrous nitrosylated neuroglobin (Ngb(II)-NO) [12].

Remarkably, the O_2-mediated oxidation of HSA-heme-Fe(II)-NO is allosterically modulated by rifampicin, an anti-tuberculosis drug binding to Sudlow's site I [22]. Values of the thermodynamic parameter L obtained by mixing HSA-heme-Fe(II)-NO with O_2 and CO are in excellent agreement (=4.4×10^{-4} and 4.6×10^{-4} M, respectively; see Figs. 1 and 2), and are about 3-fold higher than that reported for drug binding to HSA-heme-Fe(III) (=1.2×10^{-4} M) [22]. In turn, values of L for rifampicin binding to HSA-heme-Fe(II)-NO (=4.5×10^{-4} M; present study) and HSA-heme-Fe(III) (=1.2×10^{-4} M) [22] are higher by about 30- and 10-folds, respectively, than that reported for drug binding to heme-free HSA (=1.3×10^{-5} M) [22]. These findings confirm that rifampicin and heme binding sites (*i.e.*, Sudlow's site I and the FA1 pocket) are allosterically coupled. This linkage is dependent on: (i) the redox state of the heme, (ii) the type of the heme-bound ligand, and (iii) the heme-Fe-atom coordination state. In this respect, it should be outlined that HSA-heme-Fe(II)-CO is hexa-coordinated [35], whereas HSA-heme-Fe(II)-NO is penta-coordinated [29–31]; this difference might be responsible for the observed difference of the rifampicin affinity constant L.

In structural terms, Sudlow's site I ligands (such as rifampicin) interact with Tyr150 and Arg252, two key residues positioned in the center of the drug binding site. In turn, Tyr150 drives the reorientation of Phe149 that is no longer available for the stabilization of the heme-Fe porphyrin ring by π–π stacking with a consequent effect on the heme-Fe-based structural arrangement and catalysis [3,7–9,19].

In conclusion, results described here indicate that drugs could modulate HSA functions and strongly support the view that HSA acts not only as a heme carrier but also displays heme-based reactivity [9]. It should be noticed that peroxynitrite isomerization by

HSA-heme-Fe(III) could occur in patients affected with a variety of severe hematologic diseases characterized by excessive intravascular hemolysis, and appears to be modulated by drugs [8,20]. Under these pathological conditions, the HSA-heme-Fe(III) plasmatic level increases from the physiological concentration (*ca.* 1×10^{-6} M) to *ca.* 4×10^{-5} M (see [8,20]). Therefore, the higher HSA-heme concentration and the higher efficiency of peroxynitrite isomerization altogether contribute to identify HSA-heme-Fe as a major detoxification element in the bloodstream.

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