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ScienceDirect
Biochemical and Biophysical Research Communications 369 (2008) 686–691
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Abacavir and warfarin modulate allosterically kinetics of NO dissociation from ferrous nitrosylated human serum heme-albumin

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Received 13 February 2008
Available online 26 February 2008

Abstract

Human serum albumin (HSA) participates to heme scavenging, in turn HSA–heme binds gaseous diatomic ligands at the heme–Fe-atom. Here, the effect of abacavir and warfarin on denitrosylation kinetics of HSA–heme–Fe(II)–NO (i.e., k_{off}) is reported. In the absence of drugs, the value of k_{off} is $(1.3 \pm 0.2) \times 10^{-4}$ s^{-1}. Abacavir and warfarin facilitate NO dissociation from HSA–heme–Fe(II)–NO, the k_{off} value increases to $(8.6 \pm 0.9) \times 10^{-4}$ s^{-1}. From the dependence of k_{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., $K = (1.2 \pm 0.2) \times 10^{-3}$ M and $(6.2 \pm 0.7) \times 10^{-5}$ M, respectively) were determined. The increase of k_{off} values reflects the stabilization of the basic form of HSA–heme–Fe by ligands (e.g., abacavir and warfarin) that bind to Sudlow's site I. This event parallels the stabilization of the six-coordinate derivative of the HSA–heme–Fe(II)–NO atom. Present data highlight the allosteric modulation of HSA–heme–Fe(II) reactivity by heterotropic effectors.

Keywords: Ferrous nitrosylated human serum heme-albumin; Drug-dependent denitrosylation kinetics; Abacavir; Warfarin; Allostery

Human serum albumin (HSA), the most prominent protein in plasma, provides a depot and carrier for many compounds, affects pharmacokinetics of many drugs, holds some ligands in a strained orientation providing their metabolic modification, renders harmless potential toxins transporting them to disposal sites, accounts for most of the antioxidant capacity of human serum, and displays (pseudo-)enzymatic properties [1–15].

HSA displays a three-domain modular structure probably arising from duplication(s) and divergent evolution of an ancestral gene followed by a fusion event(s). Terminal regions of sequential domains contribute to the formation of flexible interdomain helices linking domain I to II, and II to III, respectively. Each domain consists of two separate sub-domains (named A and B) connected by a random coil [2,3,5,11,13,16,17].

HSA provides a variety of inter-domain and intra-domain ligand binding sites. Heme binds with $K_d = 1.0 \times 10^{-7}$ M to a site located in subdomain IB, with the tetrapyrrole ring arranged in a D-shaped cavity limited by Tyr138 and Tyr161 that provide π–π stacking interaction with the porphyrin and supply a donor oxygen (from Tyr161) coordinating the heme iron. Heme propionates point towards the interface between domains I and III and are stabilized by salt bridges with His146 and Lys190

Abbreviations: Hb, hemoglobin; HPX, hemopexin; HSA, human serum albumin; Lb, leghemoglobin; Mb, myoglobin; Ngb, neuroglobin; sGC, soluble guanylyl cyclase; trHbO, truncated Hb O; IHP, inositol hexakisphosphate; 1-MeIm, 1-methylimidazole.

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doi:10.1016/j.bbrc.2008.02.077

[18,19]. Bulky heterocyclic molecules bind preferentially to Sudlow's site I, whereas Sudlow's site II is preferred by aromatic carboxylates with an extended conformation [1–15,20].

The heme pocket and Sudlow's site I are spectroscopically and functionally coupled, indeed Sudlow's site I ligands affect allosterically heme binding and vice versa. Heme binding to HSA inhibits ligand association to Sudlow's site I by stabilizing the basic (B) state of HSA, whereas ligand association to Sudlow's site I impairs human serum heme-albumin (HSA–heme) formation by stabilizing the neutral (N) state of HSA [9–13,21–23]. Interestingly, HSA–heme binds NO and CO and exhibits catalase and peroxidase activity [11,20,24–30]. Furthermore, HSA–heme mutants have been proposed as O_2-carriers [31,32]. Remarkably, abacavir modulates allosterically kinetics of peroxynitrite-mediated oxidation of human ferrous nitrosylated HSA–heme (HSA–heme–Fe(II)–NO) [33].

Here, the effect of abacavir (an anti-retroviral drug) and warfarin (an anticoagulant medication) on denitrosylation kinetics of HSA–heme–Fe(II)–NO is reported. Abacavir and warfarin accelerate NO dissociation from HSA–heme–Fe(II)–NO, highlighting the allosteric modulation of HSA–heme–Fe(II) reactivity by heterotropic effectors which appears to be linked to the redox state of the HSA–heme–Fe atom.

Materials and methods

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

HSA–heme–Fe(II) (7.6×10^{-6} M) was prepared by adding a 1.2-molar excess of HSA to the heme–Fe(II) solution (1.0×10^{-1} M sodium phosphate buffer, pH 7.0) at 10.0 °C. HSA–heme–Fe(II)–NO (3.8×10^{-6} M) was obtained, under anaerobic conditions, by blowing purified NO over the ferrous heme–protein solution (1.0×10^{-1} M sodium phosphate buffer, pH 7.0) at 10.0 °C. Then, the excess of NO was pumped off gently before recording kinetics [20,25,27,29].

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 [34]. The abacavir stock solution (1.0×10^{-2} M) was prepared by dissolving the drug in methanol [35]. Drug stock solutions were then mixed with the HSA–heme–Fe(II)–NO (final concentration, 3.8×10^{-6} M) solution to obtain the desired final abacavir and warfarin concentration, ranging between 1.0×10^{-5} M and 5.0×10^{-3} M.

The CO solution was prepared by keeping in a closed vessel the 1.0×10^{-1} M phosphate buffer solution (pH 7.0) under CO at $P = 760.0$ mm Hg anaerobically ($T = 20.0$ °C).

Values of the first-order rate constant for NO dissociation from HSA–heme–Fe(II)–NO (i.e., for NO replacement by CO; k_{off}) were obtained by mixing the HSA–heme–Fe(II)–NO (final concentration, 3.8×10^{-6} M) solution with the CO (final concentration, 1.0×10^{-4} M to 5.0×10^{-4} M) dithionite (final concentration, 1.0×10^{-2} M) solution under anaerobic conditions, at pH 7.0 (1.0×10^{-1} M phosphate buffer) and $T = 20.0$ °C [36], in the absence and presence of abacavir and warfarin (final concentration, 1.0×10^{-5} M to 5.0×10^{-3} M). Kinetics was monitored between 360 nm and 460 nm (wavelength interval = 5 nm). Spectra were collected every 30 s.

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

Scheme 1.

The time course for HSA–heme–Fe(II)–NO denitrosylation was fitted to a single exponential process according to the minimum reaction mechanism represented by Scheme 1 [36].

Values of k_{off} were determined from data analysis according to Eq. (1) [37]:

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

Values of the dissociation equilibrium constant for drug binding to HSA–heme–Fe(II)–NO (i.e., K) were obtained from the dependence of k_{off} on the abacavir and warfarin concentration (i.e., [drug]). Values of K were determined from data analysis, according to Eq. (2) [37]:

$$k_{obs} = k_{off}^{*} \times [\text{drug}]/(K + [\text{drug}]) + k_{off}^{+} \times K/(K + [\text{drug}]) \quad (2)$$

where k_{off}^{*} is the k_{obs} value obtained in the presence of saturating amounts of abacavir or warfarin (i.e., under conditions where [drug] >> K), and k_{off}^{+} is the k_{obs} value obtained in the absence of drugs (i.e., under conditions where [drug] = 0).

Results and discussion

Under all the experimental conditions, the time course for NO dissociation from HSA–heme–Fe(II)–NO conforms to a single-exponential decay for more than 90% of its course, in the absence and presence of abacavir and warfarin (Fig. 1). Values of the first-order rate constant for NO dissociation from HSA–heme–Fe(II)–NO (i.e., k_{off}) are wavelength- and [CO]-independent in the presence of dithionite excess (data not shown).

Values of k_{off} for HSA–heme–Fe(II)–NO denitrosylation increase from $(1.3 \pm 2) \times 10^{-4}$ s^{-1}, in the absence of drugs (i.e., k_{off}^{+} in Eq. (2)), to $(8.6 \pm 0.9) \times 10^{-4}$ s^{-1}, in the presence of saturating amounts of abacavir and warfarin (i.e., k_{off}^{*} in Eq. (2)) (Fig. 1 and Table 1). This finding reflects the stabilization of the B state of HSA–heme–Fe by ligands (e.g., abacavir and warfarin) that bind to Sudlow's site I [9–13,21–23]. This event is accompanied by the stabilization of the six-coordinate derivative of the HSA–heme–Fe(II)–NO species, which is instead predominantly five-coordinated in the N state (i.e., in the absence of ligands of Sudlow's site I) [11,20,27,29].

The 6.6-fold increase of the k_{off} value for NO dissociation from HSA–heme–Fe(II)–NO upon stabilization of the six-coordinated B state by abacavir and warfarin (present study) reflects a behavior similar to that reported for the heme–Fe(II)–NO model compound, where the k_{off} value for NO dissociation increases by 1400-folds following the *trans* binding of 1-methyl-imidazole (1-MeIm) [25] (Table 1). However, the binding in *trans* of a sixth axial ligand to several ferrous nitrosylated hemoproteins is instead generally accompanied by a decrease of the NO dissociation rate constant. Thus, k_{off} values for NO dissociation from five-coordinate ferrous nitrosylated hemopro-

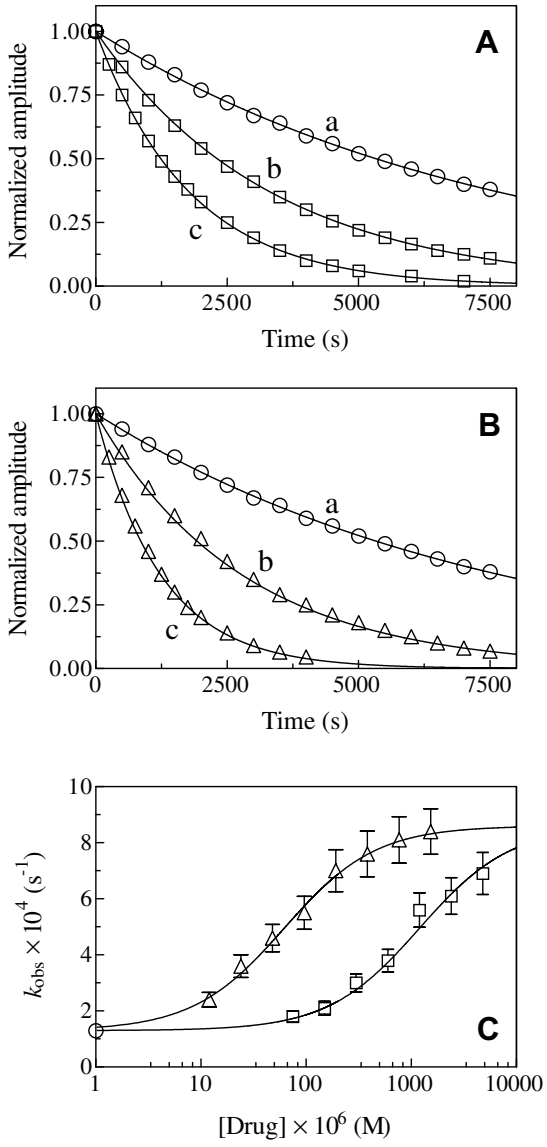

Fig. 1. Kinetics of NO dissociation from HSA–heme–Fe(II)–NO at pH 7.0 and 20.0 °C, in the absence and presence of abacavir and warfarin. (A) Normalized averaged time courses for NO dissociation from HSA–heme–Fe(II)–NO in the absence (circle) and presence (squares) of abacavir. The time course analysis according to Eq. (1) allowed to determine the following values of $k_{off} = 1.3 \times 10^{-4}\ s^{-1}$ (trace a), $3.0 \times 10^{-4}\ s^{-1}$ (trace b), and $5.6 \times 10^{-4}\ s^{-1}$ (trace c). Values of k_{obs} were obtained at [abacavir] = 0 M (trace a), 3.0×10^{-4} M (trace b), and 1.2×10^{-3} M (trace c). (B) Normalized averaged time courses for NO dissociation from HSA–heme–Fe(II)–NO in the absence (circle) and presence (triangles) of warfarin. The time course analysis according to Eq. (1) allowed to determine the following values of $k_{off} = 1.3 \times 10^{-4}\ s^{-1}$ (trace a), $3.6 \times 10^{-4}\ s^{-1}$ (trace b), and $8.1 \times 10^{-4}\ s^{-1}$ (trace c). Values of k_{obs} were obtained at [warfarin] = 0 M (trace a), 2.4×10^{-5} M, (trace b), and 7.7×10^{-4} M (trace c). (C) Dependence of k_{obs} on the abacavir (squares) and warfarin (triangles) concentration (i.e., [drug]). The circle indicates the value of k_{off} in the absence of drugs. The analysis of data according to Eq. (2) allowed to determine the following parameters: abacavir—$k_{off}^{*} = (8.6 \pm 0.9) \times 10^{-4}\ s^{-1}$, $k_{off}^{+} = (1.3 \pm 0.2) \times 10^{-4}\ s^{-1}$, and $K = (1.2 \pm 0.2) \times 10^{-3}$ M; and warfarin—$k_{off}^{*} = (8.6 \pm 0.9) \times 10^{-4}\ s^{-1}$, $k_{off}^{+} = (1.3 \pm 0.2) \times 10^{-4}\ s^{-1}$, and $K = (6.2 \pm 0.7) \times 10^{-5}$ M. Spectra were collected every 30 s. The CO concentration ranged between 1.0×10^{-4} M and 5.0×10^{-4} M. The dithionite concentration was 1.0×10^{-2} M. The HSA–heme–Fe(II)–NO concentration was 3.8×10^{-6} M.

teins are higher than those of six-coordinate species (Table 1) (see [25,38–43] and present study).

The *trans* base effect, that appears to modulate k_{off} for denitrosylation of the heme–Fe(II)–NO model compound and HSA–heme–Fe(II)–NO (Table 1), can be counterbalanced and eventually overwhelmed by multiple protein structure-dependent 'cage effects' occurring in most of the six-coordinate ferrous nitrosylated heme-proteins. In spite of the fact that the heme in HSA–heme–Fe is highly solvent accessible [18,19], as also reported for the heme–Fe model compound [25], there is a significant likelihood that, following dissociation, NO undergoes the geminate recombination with the heme–Fe(II) atom before it escapes to the surrounding solvent [25]. This phenomenon, which seems to be more evident in five-coordinated heme–Fe(II)–NO species than in six-coordinated derivatives [44], might contribute significantly to the slower NO dissociation rate constant of the five-coordinated HSA–heme–Fe(II)–NO (in the absence of drugs) with respect to the six-coordinated form (in the presence of drugs) (Table 1).

Furthermore, values of k_{off} for NO dissociation from ferrous nitrosylated heme-proteins (Table 1) reflect structurally different stabilization mode(s) of the heme–Fe(II)-bound NO by amino-acid residue(s) located in the heme distal pocket. In fact, a hydrogen bonding network involving distal Tyr and Trp residues stabilizes the heme-bound ligand in *Mycobacterium tuberculosis* truncated hemoglobin O (trHbO) [45,46]. Furthermore, Tyr145 stabilizes the heme-bound NO in soluble guanylyl cyclase (sGC) by hydrogen bonding [47]. In contrast, the stabilization of the heme-bound ligand is achieved by hydrogen bonding to the heme distal His residue in human hemoglobin (Hb), horse heart myoglobin (Mb), human neuroglobin (Ngb), and soybean leghemoglobin (Lb), and possibly in rabbit hemopexin–heme (HPX–heme) [38,39,48–53]. Lastly, Arg145 may stabilize the HSA–heme–Fe(II)–NO complex by hydrogen bonding (unpublished results).

In contrast, values of the first-order rate constant for NO dissociation from ferric nitrosylated heme-model compounds and heme-proteins are higher than those of k_{off} by at least two orders of magnitude, spanning from $1.2 \times 10^{-1}\ s^{-1}$ to $2.6 \times 10^{1}\ s^{-1}$ (see [33]), this reflects the low affinity of NO for heme–Fe(III) systems [37]. Furthermore, values of the first-order rate constant for NO dissociation from HSA–heme–Fe(III)–NO are unaffected by abacavir [33], suggesting an interplay between the allosteric modulation of HSA–heme–Fe reactivity and the redox state of the heme–Fe atom.

The analysis of the dependence of k_{off} on the drug concentration (Fig. 1) according to Eq. (2) allowed to determine values of the dissociation equilibrium constant for abacavir and warfarin binding to HSA–heme–Fe(II)–NO (i.e., $K = 1.2 \times 10^{-3}$ M and 6.2×10^{-5} M, respectively) (Fig. 1). Values of K for abacavir and warfarin binding to HSA–heme–Fe(II)–NO are about 3-fold lower than those reported for drug binding to HSA–heme–Fe(III) ($K = 4.5 \times 10^{-4}$ M and 2.1×10^{-5} M, respectively)

Table 1
Values of k_{off} for denitrosylation of some heme–Fe(II)–NO systems and heme–Fe(II)–NO coordination state

Heme–Fe(II)–NO system	Effector	k_{off} (s^{-1})[a]	Coordination
Heme–Fe[b]	—	2.1×10^{-5}	5c
	1-MeIm	2.9×10^{-2}	6c
HSA–heme–Fe[c]	—	1.3×10^{-4}	5c
	Abacavir, warfarin	8.6×10^{-4}	6c
Rabbit HPX–heme–Fe[d]	—	9.1×10^{-4}	6c
Bovine sGC[e]	—	8.2×10^{-4}	5c
Mycobacterium leprae trHbO[f]	—	1.3×10^{-4}	6c
Soybean Lb[g]	—	2.0×10^{-5}	6c
Horse heart Mb[h]	—	1.0×10^{-4}	6c
Human Ngb[i]	—	2.0×10^{-4}	6c
Human Hb, R-state, α-chains[j]	—	1.6×10^{-4}	6c
Human Hb, R-state, β-chains[j]	—	8.0×10^{-5}	6c
Human Hb, T-state, α-chains[j]	IHP	4.4×10^{-3}	5c
Human Hb, T-state, β-chains[j]	IHP	9.4×10^{-5}	6c

[a] For details, see Scheme 1.
[b] pH 7.4 and $T = 20.0$ °C. Six-coordinate heme–Fe(II)–NO was obtained by adding saturating amounts of 1-MeIm. From [25].
[c] pH 7.0 and $T = 20.0$ °C. Six-coordinate HSA–heme–Fe(II)–NO was obtained by adding saturating amounts of abacavir and warfarin. Present study.
[d] pH 7.0 and $T = 10.0$ °C. From [42].
[e] pH 7.4 and $T = 20.0$ °C. From [25].
[f] pH 7.0 and $T = 20.0$ °C. From [43].
[g] pH 7.0 and $T = 20.0$ °C. From [38,39].
[h] pH 7.4 and $T = 20.0$ °C. From [25].
[i] pH 7.5 and $T = 25.0$ °C. From [40].
[j] pH 7.2 and room temperature. The T-state of human Hb(II)–NO was obtained by adding saturating amounts of IHP. From [41].

[10,20]. In turn, values of K for abacavir and warfarin binding to HSA–heme–Fe(II)–NO (present study) and HSA–heme–Fe(III) [10,20] are lower by about 30- and 10-folds, respectively, than those reported for drug binding to heme-free HSA ($K = 4.3 \times 10^{-5}$ M and 3.0×10^{-6} M, respectively) [10,54]. These findings confirm that heme inhibits allosterically abacavir and warfarin binding to HSA [10,20,54] and highlight for the first time the role of the redox state of the HSA–heme–Fe atom on modulating drug association.

As a whole, present data indicate that HSA could be taken as the prototype of monomeric allosteric proteins [11]. Although HSA is formed by a single polypeptide chain, its three-domain modular structure, the presence of interdomain contacts, the flexibility of interdomain connections, and the presence of ligand binding sites in the contact regions endow HSA with allosteric properties, heterotropic interactions modulating the conformational transition between at least two different states [11,13]. Remarkably, HSA–heme represents a unique case within heme-proteins since allosteric effectors modulate both the affinity of the heme–Fe for the protein matrix and the heme reactivity.

Acknowledgments

This work was partially supported by grants from the Ministry for University and Research of Italy (Department of Biology, University 'Roma Tre', Roma, Italy, 'CLAR 2007') and from the Ministry of Health of Italy (National Institute for Infectious Diseases I.R.C.C.S. 'Lazzaro Spallanzani', Roma, Italy, 'Ricerca corrente 2007') to P.A.

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