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Modulation of the reactivity of the thiol of human serum albumin and its sulfenic derivative by fatty acids

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Abstract

The single cysteine residue of human serum albumin (HSA-SH) is the most abundant plasma thiol. HSA transports fatty acids (FA), a cargo that increases under conditions of diabetes, exercise or adrenergic stimulation. The stearic acid-HSA (5/1) complex reacted 6-fold faster at pH 7.4 with the disulfide 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 2-fold faster with hydrogen peroxide and peroxynitrite. The apparent pK_a of HSA-SH decreased from 7.9 ± 0.1 to 7.4 ± 0.1 . Exposure to H_2O_2 (2 mM, 5 min, 37 °C) yielded 0.29 ± 0.04 moles of sulfenic acid (HSA-SOH) per mole of FA-bound HSA. The reactivity of HSA-SOH with low molecular weight thiols increased ~3-fold in the presence of FA. The enhanced reactivity of the albumin thiol at neutral pH upon FA binding can be rationalized by considering that the corresponding conformational changes that increase thiol exposure both increase the availability of the thiolate due to a lower apparent pK_a and also loosen steric constraints for reactions. Since situations that increase circulating FA are associated with oxidative stress, this increased reactivity of HSA-SH could assist in oxidant removal.

Keywords

Human serum albumin; Thiol; Sulfenic acid; Sulfinic acid; Fatty acids

INTRODUCTION

Human serum albumin (HSA)¹ is the most abundant protein in plasma (~0.6 mM). It is a 66.5 kDa protein that displays several physiological functions. It participates in the

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maintenance of colloid osmotic blood pressure and it binds and transports fatty acids (FA), hormones, bilirubin, vitamins, metal cations and drugs [1]. Also, HSA has a recognized antioxidant role in plasma, scavenging oxidant species [2].

HSA plays a key role in lipid metabolism, acting as transporter between plasma, lipoproteins and tissues. This role is underscored by the fact that patients lacking HSA suffer deep disturbances in lipid metabolism [3]. Circulating HSA carries around 0.3–1 fatty acids per albumin molecule (FA/HSA) [4]. This molar ratio may increase to 4–6 FA/HSA under adrenergic stimulation, exercise and some pathologies like diabetes mellitus [1]. Several binding sites for FA have been characterized, with the strongest ones displaying affinity constants of $\sim 10^8 \text{ M}^{-1}$ [5]. No two FA-binding sites are identical in detail, although each comprises a hydrophobic pocket that interacts with the hydrocarbon chain and is capped at one end with basic or polar residues that interact with the carboxylate [5, 6]. Binding of FA is associated with significant structural changes in the albumin molecule [6] which determine changes in the physical chemical properties, such as an increase in the stability towards heat and proteases [1].

HSA contains 585 amino acids, among which 35 are cysteine residues that form 17 disulfide bridges [1]. The single free cysteine left, Cys34, accounts for $\sim 80\%$ of the total free thiols in plasma. About 70 % of circulating HSA contains Cys34 in the reduced thiol state (HSA-SH), while the rest consists mostly of mixed disulfides with cysteine and other low molecular weight thiols [7–9]. A minor fraction is oxidized to higher oxidation states such as sulfinic acid (HSA-SO₂H) and sulfonic acid (HSA-SO₃H). The oxidized forms of HSA are increased under certain conditions and pathological situations, and are also present in preparations intended for clinical use (reviewed in [10]).

Previous work with FA-free HSA showed that the two-electron oxidation of the thiol yields a relatively stable sulfenic acid (HSA-SOH) [2, 11, 12]. Recently, using its reaction with thionitrobenzoate (TNB), we were able to obtain quantitative information on HSA-SOH and determined the rate constants of several of its reactions [13]. HSA-SOH can have three possible fates. It can react with low molecular weight thiols yielding mixed disulfides, it can react with a second oxidant forming sulfinic acid or it can decompose spontaneously to an incompletely characterized species, HSA-SX. The formation of intermolecular disulfide HSA dimers is not observed, probably due to steric limitations.

Although the Cys34 residue is not directly involved in the FA-binding sites, its oxidizability is intimately coupled to the binding of FA [14–16]. As continuation of our previous work, we studied changes in the properties of the thiol and the sulfenic derivative that occur upon FA binding.

MATERIALS AND METHODS

Materials

HSA, FA sodium salts and all remaining reagents were from Sigma. Absorbance determinations were made in a Varian Cary 50 spectrophotometer. For rapid kinetics, an Applied Photophysics RX2000 stopped flow accessory was connected to the spectrophotometer or, alternatively, an Applied Photophysics SF17MV instrument was used.

¹**Abbreviations used:** HSA, human serum albumin; HSA-SH, thiol in human serum albumin; SH/HSA, mol of thiol per mol of HSA; HSA-SOH, sulfenic acid in human serum albumin; HSA-SO₂H, sulfinic acid in human serum albumin; FA, fatty acid; FA/HSA, mol of FA per mol of HSA; DTNB, 5,5'-dithiobis(2-nitrobenzoate); TNB, 5-thio-2-nitrobenzoate; DTPA, diethylenetriaminepentaacetic acid; *cis*-parinaric acid, 9,11,13,15-*cis-trans-trans-cis*-octadecatetraenoic acid.

Solutions

All assays, unless specified, were performed in 100 mM phosphate buffer, pH 7.4, containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA). FA solutions (200 mM) were freshly prepared in methanol previous to each HSA lipitation step. Complete solubility was accomplished at ~80 °C with agitation. Hydrogen peroxide stock solution (0.57 M) was prepared in nanopure water and its concentration was determined spectrophotometrically at 240 nm ($43.6 \text{ M}^{-1} \text{ cm}^{-1}$ [17]). Catalase was prepared in phosphate buffer and the apparent first-order rate constant of 10 mM hydrogen peroxide decay was determined to estimate its concentration [18]. Peroxynitrite (ONOO^-) was synthesized from hydrogen peroxide and nitrite [19], stored at -80°C and diluted in NaOH (10 mM). Peroxynitrite concentration was determined from the absorbance at 302 nm in 1 M NaOH ($1670 \text{ M}^{-1} \text{ cm}^{-1}$ [20]). TNB, free of 5,5'-dithiobis(2-nitrobenzoate) (DTNB), was prepared by reduction of DTNB with 2-mercaptoethanol followed by ion exchange chromatography [13].

HSA solutions

HSA contains traces of lipids and other contaminants, therefore it was necessary to delipidate the protein and to lipidate it again under controlled conditions. HSA was defatted with activated charcoal [21]. The thiol was reduced by 15 min incubation with 2-mercaptoethanol (10 mM, room temperature) followed by gel filtration on PD-10 columns (GE Healthcare) equilibrated with phosphate buffer. The HSA concentration was determined from the absorbance at 279 nm ($0.531 (\text{g/L})^{-1} \text{ cm}^{-1}$) considering a molecular mass of 66438 Da [1]. Thiol concentration was measured with DTNB after 5 min incubation with excess reagent in sodium pyrophosphate buffer (0.1 M, pH 9). Free TNB formed was quantified at 412 nm ($14150 \text{ M}^{-1} \text{ cm}^{-1}$ [22]). Reduced HSA solutions thus prepared had a concentration of ~1 mM and typically 0.7–0.9 SH/HSA. The lipitation process consisted of incubating previously delipidated and reduced HSA with a sufficient amount of a 200 mM FA methanolic solution for the corresponding 5/1 FA/HSA molar ratio during one hour, under agitation, at room temperature. Finally, FA-bound HSA solutions were gel filtered. Reported studies based on gas-chromatography and exchange dialysis verify the FA/HSA expected molar ratio [23–25]. To obtain preparations enriched in HSA-SOH, reduced FA-free or FA-bound HSA (0.25 mM) was incubated with 2 mM hydrogen peroxide for 5 min at 37°C in phosphate buffer. Reactions were stopped by the addition of enough catalase to consume 95 % of the remaining hydrogen peroxide in 5 s. Solutions were maintained on ice and used within the same day. Fractions oxidized to sulfinic acid (HSA-SO₂H) were prepared by incubation with 10 mM hydrogen peroxide for 10 min at 37°C . For some experiments, the thiol and sulfinic acid forms were purified by chromatography using a weak anion exchange column (TSK DEAE 5PW glass $7.5 \text{ cm} \times 8 \text{ mm}$ I.D., $10 \mu\text{m}$, Tosoh Bioscience LLC, Tokyo, Japan) preequilibrated with buffer A (10 mM ammonium acetate adjusted to pH 5.35 with glacial acetic acid). The elution was performed with a linear gradient of buffer B (similar as buffer A, but adjusted to pH 3.95) at a flow-rate of 1 mL/min. Under these conditions, the different HSA fractions can be separated (H. Botti, manuscript in preparation).

Reaction between the albumin thiol and DTNB

Reduced FA-free or FA-bound HSA ($10 \mu\text{M}$) was mixed with increasing concentrations of DTNB ($75\text{--}600 \mu\text{M}$) in phosphate buffer using a stopped flow accessory and the absorbance at 412 nm was recorded for about one hour at 25°C . Time courses were fitted to single-exponential equations to determine the pseudo-first-order rate constants (k_{obs}).

pH dependency of the reactions of the albumin thiol with hydrogen peroxide and DTNB

To study the reaction between HSA-SH and hydrogen peroxide, reduced FA-free or FA-bound HSA (100 μ M), prepared in phosphate buffer (10 mM, pH 7.4, 0.1 mM DTPA) was mixed with hydrogen peroxide (100 μ M) in the presence of constant ionic strength buffers of varying pHs consisting of MES (0.1 M), Tris (0.052 M) and ethanolamine (0.052 M) [26], at 37 °C. The decrease in hydrogen peroxide concentration was determined using a specific electrode (ISO-HPO-2 sensor) attached to an APOLLO 4000 analyzer (World Precision Instruments). Current values were compared with calibration curves performed at each pH. The initial rate of the reaction was determined while the current changed linearly with time. To study the pH dependency of the reaction with DTNB, reduced HSA (20 μ M FA-free or 10 μ M FA-bound) in the constant ionic strength buffer (MES-Tris-ethanolamine) was mixed with DTNB (100 μ M or 50 μ M respectively), and the initial rate of the reaction was determined by the increment in the absorbance at 412 nm due to TNB formation, at 25 °C. Values were corrected for the fraction of TNB that could not be observed because of protonation at the more acidic pHs (pK_a 4.4, [27] and our own determination). Second-order rate constants were determined by dividing the initial rate by HSA-SH and oxidant concentrations.

Reaction between the albumin thiol and peroxynitrite

In the stopped flow, peroxynitrite (15 μ M final concentration) was mixed with increasing concentrations of reduced FA-free or FA-bound HSA (~100–280 μ M) in phosphate buffer. The absorbance at 302 nm was recorded for ~10 s at 37 °C.

Reaction between the albumin sulfenic derivative and TNB

FA-free or FA-bound HSA (0.25 mM) was oxidized with H_2O_2 (2 mM, 5 min, 37 °C) and the reactions were stopped with catalase. Aliquots (25 μ M) were mixed with TNB (30–70 μ M) using a stopped flow accessory and the decrease in TNB concentration was recorded at 412 nm, 25 °C, pH 7.4 [13].

Reaction between the albumin sulfenic derivative and hydrogen peroxide

Time courses of HSA-SOH formation and decay for FA-free and FA-bound HSA were followed using the reaction with TNB [13]. Reduced HSA (0.25 mM) was incubated with hydrogen peroxide (2 mM) at 37 °C. At increasing times, aliquots (50 μ M FA-free or 25 μ M FA-bound HSA) were mixed with catalase (in sufficient amount to consume 95 % of the remaining H_2O_2 in 5 s) and TNB (70 μ M or 35 μ M for FA-free and FA-bound HSA, respectively) at 25 °C in phosphate buffer. The absorbance at 412 nm was recorded for 1 min and the initial rate of the absorbance decay was determined to evaluate the HSA-SOH concentration [13].

Stability of albumin sulfenic acid in phosphate buffer

Oxidized (2 mM H_2O_2 , 5 min, 37 °C) FA-free or FA-bound HSA (0.25 mM) was incubated at 37 °C in phosphate buffer. At increasing times, aliquots (50 μ M FA-free or 25 μ M FA-bound HSA) were mixed with TNB (70 μ M or 35 μ M, respectively) and the initial rate of the absorbance decay at 412 nm and 25 °C, indicative of HSA-SOH concentration, was measured.

Reaction of albumin sulfenic acid with thiols

The rate constants of the reactions of FA-free and FA-bound HSA-SOH with different thiols were determined using a competition assay with TNB [13]. Using stopped flow, aliquots (50 μ M FA-free or 25 μ M FA-bound) of oxidized HSA (2 mM H_2O_2 , 5 min, 37 °C) were mixed with TNB (70 or 35 μ M, respectively) at 25 °C in the absence or presence of increasing

concentrations of glutathione (0.25–3.0 mM), cysteine (40.6–406 μ M), homocysteine (190–524 μ M) or cysteinylglycine (26–140), and the absorbance at 412 nm was recorded for ~10 min. Thiol concentration was measured before and after the experiment.

Binding of *cis*-parinaric acid to HSA

The stock solution of 9,11,13,15-*cis-trans-trans-cis*-octadecatetraenoic acid (*cis*-parinaric acid) was prepared by the addition of 10 mg of the solid to 1 mL ethanol and 120 μ g/mL 2,6-di-*tert*-butyl-4-methylphenol (BHT) under argon, stored at -80°C and protected from light [28]. The concentration of working solutions was determined from the absorbance at 305 nm ($74200\text{ M}^{-1}\text{ cm}^{-1}$ [29] in ethanol). FA-free HSA, either reduced or oxidized to sulfinic acid (10 mM H_2O_2 , 10 min, 37°C), was purified by ion-exchange chromatography as described above. Increasing concentrations of both albumins were incubated with 2 μ M *cis*-parinaric acid in phosphate buffer for ~10 min at 25°C to allow the mixtures to reach equilibrium, and fluorescence was read at 420 nm with excitation at 320 nm in a Varian Cary Eclipse fluorometer.

Data Processing

Data were plotted and analyzed using OriginPro 8.0 (Microcal Software). Unless specified, expressed errors represent either the standard deviation of 3 repetitions or the parameter error of the fit. The reported crystallographic structures were analyzed using PyMOL v0.99 (<http://www.pymol.org> [30]). Calculations to determine the surface accessibility of Cys34 were made with ASAView (<http://www.netasa.org/asaview> [31]). Dynafit v. 3.28.070 [32] was used for the *cis*-parinaric acid binding data fitting.

RESULTS

Reactivity of the albumin thiol with DTNB

The reactivity of HSA complexed with different FA in 5/1 FA/HSA molar ratios towards the low molecular weight disulfide DTNB was studied as a first approach to understand the changes that occur in the properties of HSA-SH upon FA binding. The reaction between HSA-SH and DTNB can be written as follows:

When reduced HSA was mixed with DTNB in excess at pH 7.4, the appearance of the product TNB increased with time as expected for a pseudo-first order process (Fig. 1A). At increasing DTNB concentrations, a proportional increase in k_{obs} was observed while the amplitude did not change (Fig. 1B), confirming that the reaction was also first-order in DTNB. No saturation behavior was observed and the *y*-intercept was close to zero, suggesting that the reaction occurred under irreversible conditions. From the slopes of k_{obs} versus DTNB concentration plots, the second-order rate constants at pH 7.4 were determined. The rate constant for FA-free HSA was $16.1 \pm 0.5\text{ M}^{-1}\text{ s}^{-1}$ at a ionic strength of ~0.26 M. This agrees with the previous determination of $14.8 \pm 0.8\text{ M}^{-1}\text{ s}^{-1}$ under similar conditions [13]. For the 5/1 complexes with saturated FA of increasing chain length, as exemplified for palmitic acid in Fig. 1, the rate constants increased ~six-fold. Thus, for lauric acid (12:0) the rate constant was $95 \pm 5\text{ M}^{-1}\text{ s}^{-1}$; for myristic acid (14:0), $117 \pm 5\text{ M}^{-1}\text{ s}^{-1}$; for palmitic acid (16:0), $90 \pm 2\text{ M}^{-1}\text{ s}^{-1}$ and for stearic acid (18:0), $113 \pm 2\text{ M}^{-1}\text{ s}^{-1}$. We also assayed the oleic acid (18:1 Δ^9) 5/1 complex, to see the effect of an unsaturation in the carbon chain but, at $102\text{ M}^{-1}\text{ s}^{-1}$, it also reacted six-fold faster than FA-free HSA. Polyunsaturated FA were specifically avoided because of their oxidizability. Overall, our

results agree with previous reports regarding the increase in thiol susceptibility to DTNB in the presence of FA [14, 15]. Here, second-order rate constants are reported.

On the basis of the results obtained with different complexes, we decided to continue our studies with the 5/1 stearic acid-HSA complex as a model of FA-bound HSA. From now on, descriptions of experiments in which FA-bound HSA is compared to FA-free HSA refer to this particular complex.

Reactivity of the albumin thiol with hydrogen peroxide

The HSA thiol can react with hydrogen peroxide giving the sulfenic derivative (HSA-SOH):

The reactions of FA-free or FA-bound HSA with hydrogen peroxide were studied through an initial rate approach, by following the decay of hydrogen peroxide with a specific electrode (Fig. 2). The second-order rate constants for the reaction between HSA-SH and hydrogen peroxide were $1.4 \pm 0.3 \text{ M}^{-1} \text{ s}^{-1}$ and $2.7 \pm 0.8 \text{ M}^{-1} \text{ s}^{-1}$ in the absence and presence of FA, respectively, at pH 7.4 and 37 °C. Thus, the reactivity of the thiol towards hydrogen peroxide increased two-fold in the presence of FA.

Reactivity of the albumin thiol with peroxynitrite

In the absence of HSA, peroxynitrite decayed exponentially with a k_{obs} of $1.2 \pm 0.1 \text{ s}^{-1}$ at pH 7.4 and 37 °C, consistent with previous reports [33]. In the presence of HSA, k_{obs} increased linearly (Fig. 3), in accordance with the reaction of the thiolate with peroxynitrous acid:

The second-order rate constants were $(7.5 \pm 0.3) \times 10^3$ and $(14.7 \pm 0.6) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (37 °C, pH 7.4) for FA-free and FA-bound HSA, respectively. The value obtained for FA-free HSA is consistent with our previous report of $(8.3 \pm 0.3) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ [33]. Similarly as in the case of hydrogen peroxide, the reaction with peroxynitrite was twice as fast in the presence of FA.

pH dependency of the reactions with hydrogen peroxide and DTNB

The apparent rate constants of the reaction between HSA-SH and hydrogen peroxide increased at alkaline pH, as expected for the thiolate being the reactive species (eq. 2) and considering that hydrogen peroxide does not ionize throughout the pH range tested ($\text{p}K_{\text{a}}$ 11.7). The $\text{p}K_{\text{a}}$ values of HSA-SH were 7.9 ± 0.1 and 7.4 ± 0.1 for FA-free and FA-bound HSA, respectively (Fig. 4A). From the plateau at alkaline pH, the pH-independent second-order rate constants for the reactions of the thiolate with hydrogen peroxide were determined as 5.3 ± 0.2 and $4.0 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$ in the absence and presence of FA, respectively (Fig. 4A).

In the case of DTNB (Fig. 4B), $\text{p}K_{\text{a}}$ values of 7.8 ± 0.1 and 7.1 ± 0.1 were determined for FA-free and FA-bound HSA, and the pH-independent rate constants for the thiolate were 40 ± 2 and $45 \pm 1 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The rate constants were lower in the constant ionic strength ($\mu = 0.10 \text{ M}$) buffer consisting of MES (0.1 M), Tris (0.052 M) and ethanolamine (0.052 M) used for pH dependency studies (Fig. 4B) than in the 0.1 M phosphate buffer ($\mu \sim 0.26 \text{ M}$) previously used (Fig. 1). This can be explained in terms of ionic strength effects, since HSA has about 15–19 negative charges at pH 7.4 [1] and DTNB has 2 negative charges (Fig. S1).

Formation of the HSA sulfenic derivative and its reactivity with TNB

The reaction with TNB has been used before to obtain quantitative information about sulfenic acid in FA-free HSA [13]. According to the second-order rate constant of $2.7 \pm 0.8 \text{ M}^{-1} \text{ s}^{-1}$ determined above for the reaction of FA-bound HSA-SH with hydrogen peroxide, preparations enriched in HSA-SOH were prepared by incubation with 2 mM hydrogen peroxide for 5 min at 37 °C, followed by catalase addition. Aliquots were then mixed with TNB at concentrations that represented pseudo-first-order excess with respect to HSA-SOH. Time courses (5–10 min) of TNB decay fitted an exponential plus straight line function (Fig. 5A), where the exponential term represents the following reaction [13]:

The observed rate constants increased linearly with TNB concentration (Fig. 5B) and the second-order rate constants were $119 \pm 18 \text{ M}^{-1} \text{ s}^{-1}$ for FA-free HSA, which is in accordance to the value previously reported of $105 \pm 11 \text{ M}^{-1} \text{ s}^{-1}$ [13], and $502 \pm 54 \text{ M}^{-1} \text{ s}^{-1}$ for FA-bound HSA. Considering that the amplitude of the exponential function represents the concentration of previously formed sulfenic acid, from the average value of different time courses we were able to determine the percentage of HSA that was oxidized to sulfenic acid in our conditions. For FA-free HSA, $19 \pm 1 \%$, consistent with $18 \pm 2 \%$ previously reported [13]. In the case of FA-bound HSA, the yield of sulfenic acid increased to $29 \pm 4 \%$.

Kinetic traces obtained for ~140 min fitted a biexponential equation (data not shown). The second-order rate constant of the second phase doubled in the presence of FA, from 4.7 to $10.2 \text{ M}^{-1} \text{ s}^{-1}$.

Reactivity of the albumin sulfenic derivative with hydrogen peroxide

Reduced FA-free or FA-bound HSA (0.25 mM) was incubated with hydrogen peroxide (2 mM, 37 °C). After treatment with catalase, the initial rate of TNB consumption, proportional to HSA-SOH concentration, was measured at several time points. As shown in Fig. 6, the initial rate first increased with time due to HSA-SOH formation, but then decreased due to spontaneous decay and further oxidation of HSA-SOH with excess hydrogen peroxide. The plot of the initial rate *versus* time of incubation with hydrogen peroxide fitted a biexponential function, in agreement with a mechanism involving consecutive processes with HSA-SOH as intermediate. From the ascending first phase of the plot, the second-order rate constants for the reaction of HSA-SH with hydrogen peroxide yielding HSA-SOH were 1.5 ± 0.4 and $3.0 \pm 0.8 \text{ M}^{-1} \text{ s}^{-1}$ for FA-free and FA-bound HSA, respectively, in accordance with the values determined using the hydrogen peroxide sensor and confirming by a different technique the two-fold increment.

The descending second phase observed in the bell-shaped plot in Fig. 6 is due to the contribution of two processes: the spontaneous decay of HSA-SOH to HSA-SX and the further oxidation with hydrogen peroxide to HSA-SO₂H. Subtracting the contribution of the first process (Fig. S2, see next section), calculations considering the concentration of hydrogen peroxide (2 mM) yielded second-order rate constants for the reaction between HSA-SOH and hydrogen peroxide of $0.5 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$ for FA-free, and $0.4 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$ for FA-bound HSA (37 °C, pH 7.4).

Stability of the sulfenic derivative

The HSA sulfenic derivative decays in phosphate buffer at 37 °C [13] forming HSA-SX:

We followed the same procedure as for FA-free HSA [13]. Oxidized HSA (2 mM H₂O₂, 5 min, 37 °C) was incubated in phosphate buffer at 37 °C, and aliquots were mixed with TNB at increasing times. The initial rate of the reaction with TNB, proportional to the concentration of remaining HSA-SOH, decreased exponentially with the time of incubation (Fig. S2). The first-order rate constant for HSA-SOH decay in FA-free HSA was $1.4 \times 10^{-3} \text{ s}^{-1}$, which agrees with the value previously reported of $(1.7 \pm 0.3) \times 10^{-3} \text{ s}^{-1}$ [13]. The corresponding value in the presence of FA was $(1.2 \pm 0.3) \times 10^{-3} \text{ s}^{-1}$. Thus, no significant differences were detected in the rate of spontaneous decay.

Analogously as in the case of FA-free HSA [11, 13], intermolecular disulfide or thiosulfinate dimers were not formed in FA-bound HSA according to SDS-PAGE (Fig. S3).

Reactivity of the sulfenic derivative with biologically relevant thiols

In order to study the reactivity of the sulfenic derivative with thiols, we did competition assays with TNB, as previously done for FA-free HSA [13]. FA-free or FA-bound oxidized HSA (2 mM H₂O₂, 5 min, 37 °C) was mixed with TNB and increasing concentrations of thiols that are usually present in plasma, such as cysteine, glutathione, homocysteine, or cysteinylglycine. Time courses of TNB concentration followed exponential plus straight line functions (Fig. 7A). The obtained k_{obs} were then plotted against the thiol concentration so as to determine the second-order rate constants. The amplitude of the exponential term decreased hyperbolically in the presence of the thiol, as expected for competing reactions [34]. Fig. 7B exemplifies the data with cysteinylglycine. The second-order rate constants increased by a factor of ~three in FA-bound HSA, from 21.6 ± 0.2 to $69 \pm 7 \text{ M}^{-1} \text{ s}^{-1}$ in the case of cysteine; from 9.3 ± 0.9 to $27 \pm 5 \text{ M}^{-1} \text{ s}^{-1}$ in the case of homocysteine; from 58 ± 12 to $131 \pm 1 \text{ M}^{-1} \text{ s}^{-1}$ in the case of cysteinylglycine and from 2.9 ± 0.5 to $9 \pm 2 \text{ M}^{-1} \text{ s}^{-1}$ in the case of glutathione (pH 7.4, 25 °C). It is worth noting that, in all cases, the second phase of the reaction between HSA-SOH and the different thiols presented a positive slope (Fig. 7A). This phenomenon had already been reported for FA-free HSA and homocysteine [13], and reflects the tendency of the thiolates to displace the more acidic TNB from the mixed HSA-S-TNB disulfide.

Effect of thiol oxidation in the fatty acid binding capacity of HSA

Seeking to study the subject from a different perspective, we investigated whether the oxidation state of the thiol had an effect on the capacity of the protein to bind FA. We compared reduced HSA with HSA oxidized to sulfinic acid (HSA-SO₂H) in the ability to bind *cis*-parinaric acid, a naturally occurring 18-carbon FA that is used as a biophysical probe due to the fact that its fluorescence increases when bound. Its binding to bovine [35] and human [36] albumin have been previously studied. *Cis*-parinaric acid binds reversibly to albumin with affinities similar to those of other long-chain FA.

Binding to reduced or oxidized HSA was studied by measuring the change in fluorescence of a fixed concentration (2 μM) of *cis*-parinaric in the presence of different HSA concentrations (up to 64 μM). The increase in fluorescence of *cis*-parinaric acid (Fig. 8) fitted a five-step consecutive equilibria model with dissociation constants ranging from 10^{-6} to 10^{-9} M , consistent with reported values [5, 37]. Reduced and oxidized HSA showed no significant differences in the pattern of *cis*-parinaric binding, thus, no apparent modulatory effect of HSA-SH oxidation to HSA-SO₂H on FA-binding capacity could be detected.

Analysis of the crystallographic structures

The reported crystallographic structures of HSA show that, in the absence of FA, Cys34 is buried in a shallow crevice, ~9.5 Å deep, so that the thiol is submerged in the tertiary structure of the protein (Fig. 9). Binding of FA induces conformational changes in the

protein that result in the opening of the crevice that contains Cys34, increasing the exposure of the thiol [10, 38]. Indeed, the surface accessibility of Cys34 increases from 0.7 % in FA-free HSA, to 4.9 % in HSA complexed with stearic acid.

We also analyzed the electrostatic microenvironment of the thiol by measuring the interatomic distances from the sulfur to neighbouring polar and ionizable groups. As illustrated in Fig. 9 (right panel), in FA-free HSA the distance to the hydroxyl oxygen of Tyr84 was 3.1 ± 0.2 Å, to the carboxylic oxygens of Asp38, 4.5 ± 0.3 and 6.0 ± 0.1 Å, and to the nitrogen atoms of the His39 imidazole, 4.2 ± 0.3 and 4.4 ± 0.2 Å. When the protein was complexed with stearic acid or palmitic acid, most distances were incremented, to 3.33 ± 0.07 Å (Tyr84), 4.4 ± 0.1 and 6.3 ± 0.2 Å (Asp38), and 5.32 ± 0.08 and 5.53 ± 0.08 Å (His39). These values represent the average $\pm$ standard error of the distances measured for the structures 1AO6 and 1BM0 (chains A and B) in the case of FA-free HSA and for the structures IE7H (palmitic acid) and IE7I (stearic acid), in the case of FA-bound HSA[39, 40]. In addition, it is worth noting that both in FA-free and FA-bound HSA, Cys34 is located in the N-terminus of an α -helix extending from residues 37 to 56.

DISCUSSION

The reactivity of HSA-SH presented significant differences in the presence of FA. Taking into account that complexes with $<1/1$ and $5/1$ molar ratios represent the FA/HSA normal cargo along with its reported increment under different stimuli [1], the complexes assayed constitute appropriate physiological models. At the physiological pH of 7.4, the rate constant of the reaction of the stearic acid-HSA (5/1) complex with DTNB increased six-fold. In contrast, the rate constants with hydrogen peroxide and peroxyntirite increased two-fold.

The reacting species towards disulfides and peroxides is the thiolate. Can the increased reactivity at pH 7.4 in the presence of FA be explained only on the basis of an increase in the acidity of the thiol? If so, considering that a lower pK_a value implies that a higher proportion of the thiolate is present at a certain pH, the rate constants for the reactions with different molecules should be modified by correspondingly the same factor upon fatty acid binding, reflecting the increased proportion of thiolate at that pH. Since at pH 7.4 the reactivity increased by a factor of six with DTNB but only by a factor of two with hydrogen peroxide and peroxyntirite, intrinsic changes in other molecular properties of HSA-SH should be invoked to explain the effect of FA, in addition to changes in the pK_a of the thiol.

According to the pH dependency of the reaction with hydrogen peroxide, a small and neutral molecule that is unlikely to react inespecifically, the apparent pK_a for the FA-free HSA thiol was 7.9 ± 0.1 . This value is in accordance with previous reports of ~ 8 [33, 41] and disagrees with reports of more acidic values [27, 42]. In the presence of FA the apparent pK_a of the thiol decreased by ~ 0.5 pH units. This decrease means that the conformational alterations triggered by FA induce a stabilization of ~ 0.7 kcal mol⁻¹ in the thiolate with respect to the thiol. It is likely that the decrease in apparent pK_a is mediated by the increased exposure of the Cys34 residue (Fig. 9). Upon FA binding, the environment of the thiol group would become more polar, which would tend to stabilize the thiolate. In addition, although the relative distances of the sulfur to the Tyr84, Asp38 and His39 change, and there is evidence that Tyr84 and His39 are critical since site-directed mutants present higher pK_a values [42], the effect of this altered microenvironment on the pK_a cannot be easily rationalized.

At pH 7.4, in the presence of FA there is two-fold more thiolate available for reactions, since the proportion of thiolate represents 24 % total thiol in the absence of FA (pK_a 7.9) but 50 % in their presence (pK_a 7.4). However, the stabilization of the thiolate that takes place upon FA binding, which is reflected in the decrease in pK_a , appears to work against its intrinsic

reactivity. Thus, the pH-independent rate constant for the reaction of the thiolate with hydrogen peroxide decreased from $5.3 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$ in the absence of FA to $4.0 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$ in their presence, indicating the lower reactivity of the thiolate in FA-bound HSA despite its increased abundance at pH 7.4. In contrast, in the case of DTNB, the intrinsic reactivity of the thiolate did not diminish in the presence of FA as for hydrogen peroxide, but actually increased from 40 ± 2 to $45 \pm 1 \text{ M}^{-1} \text{ s}^{-1}$. This can be taken as evidence that steric constraints are in part limiting the reactivity of the HSA thiolate and that these constraints are loosened in the presence of FA. Steric restrictions are likely to be more important in the case of DTNB, a considerably large molecule (MW = 396 g/mol), than in the case of hydrogen peroxide, which is a small molecule (MW = 34 g/mol). It is worth noting that what mediates a faster reaction of the thiolate upon FA binding is not an increased ability of the oxidants to reach the thiolate, since these reactions are not diffusion-limited, but a decrease in steric impediments allowing a better geometric alignment of the reactants. In FA-free HSA it is likely that the thiol location in a crevice prevents the appropriate alignment of atoms for reaction, and that these constraints are partly released upon FA binding. Accordingly, the surface exposure of the Cys34 is significantly incremented in the presence of FA, providing a structural basis for our argument (Fig. 9). The importance of steric constraints in limiting the reactivity of HSA-SH can also be evidenced by comparison with low molecular weight thiols. With hydrogen peroxide, low molecular weight thiolates ($7.9 \text{ p}K_a$ 9.5) react at $18\text{--}26 \text{ M}^{-1} \text{ s}^{-1}$ [43], four-fold faster than HSA-S[−], that reacts at $5.3 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 4). In contrast, with DTNB, low molecular thiolates ($7.3 \text{ p}K_a$ 8.6) react at $3\text{--}15 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ [44, 45]. This represents a thousand-fold increase with respect to HSA-S[−], that reacts at $40 \pm 2 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 4), underscoring how the reactivity of the HSA thiol is restricted by the protein environment.

The trend for increased reactivity in the presence of FA was also observed for the sulfenic derivative. Thus, at pH 7.4, the reaction of HSA-SOH with different low molecular weight thiols usually present in plasma, the reactivity increased three-fold, probably due to the more exposed orientation of Cys34. The reactivity with hydrogen peroxide, however, was not significantly affected by the presence of FA, neither was the spontaneous decay. The fact that more sulfenic acid could be detected for FA-bound than for FA-free HSA (0.29 ± 0.04 versus 0.19 ± 0.01 HSA-SOH per HSA, respectively) can be explained by considering that the rate of formation was two-fold higher in the presence of FA while the rate of decay did not change.

To extrapolate our study to a physiological context, it is first necessary to point out that, at a concentration of $\sim 450 \text{ }\mu\text{M}$, HSA-SH stands as a key oxidant scavenger, since, in contrast to the situation in intracellular compartments, antioxidant defenses in plasma are scarce and the concentration of low molecular weight thiols is $15\text{--}20 \text{ }\mu\text{M}$. This scavenger role of albumin is made evident by the fact that oxidized forms are present in the circulation (reviewed in [10, 46]). Another possible plasma antioxidant could be glutathione peroxidase, although the low concentrations of the enzyme and its substrate glutathione cast doubts on its significance [47]. It is important to consider that diffusion of oxidants into the erythrocyte and consumption by antioxidant systems therein can compete with albumin scavenging in plasma [47], with the outcome likely depending on the nature, diffusion properties and amount of the oxidant species involved. For example, on our hands, erythrocytes largely ($\sim 75 \%$) protected HSA-SH from oxidation by hydrogen peroxide (1.5 mM) but much less ($\sim 27 \%$) when peroxynitrite was the oxidant [10]. In this scenario, the increase in reactivity of HSA-SH that occurs upon FA binding, as described in this study, could be of physiological relevance particularly in the case of relatively large oxidants whose reactivity towards HSA-SH may increase several-fold and in the case of oxidants with limited diffusion into the erythrocyte. Considering that situations which tend to increase the amount of FA bound to HSA (such as adrenergic stimulation, exercise and diabetes mellitus) are

associated with oxidative stress [48, 49], a better antioxidant ability of HSA could constitute a protective adaptation. And indeed, oxidized forms of HSA are found increased under these situations [50–52]. Furthermore, the fact that FA-bound HSA-SOH reacted three-fold faster than FA-free HSA with low molecular weight thiols could impact in the final fate of the sulfenic acid, tending to increase the relative yield of mixed disulfides, which can be considered reversible intermediates, in comparison to higher oxidation states such as sulfinic and sulfonic acids, which are usually irreversible products. On the other hand, we did not find a correlation between the oxidation state of the HSA single thiol and the capacity of the protein to bind FA (Fig. 8). Thus, despite being HSA-SH more oxidizable in the presence of FA, its oxidation to sulfinic acid would not affect the capacity of the protein to bind FA. We may hypothesize that even under conditions of oxidative stress the binding/transport of FA by HSA would not be affected.

Overall, our results provide for the first time a mechanistic basis for the increased reactivity of the HSA thiol in the presence of FA, which is supported by the available structural data. The binding of FA triggers a conformational change that increases the exposure of HSA-SH and decreases its pK_a by ~0.5 pH units. Thus, the increased reactivity of the HSA thiol at neutral pH can be rationalized by increased availability of the thiolate species along with decreased steric restrictions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Changes in the reactivity and pK_a of the albumin thiol acid upon fatty acid binding were investigated.
- In the presence of fatty acids, HSA-SH reacted six-fold faster with the disulfide DTNB.
- HSA-SH reacted two-fold faster with hydrogen peroxide and peroxynitrite at neutral pH.
- The sulfenic acid of HSA was three-fold more reactive in the presence of fatty acids.
- The increased reactivity of HSA-SH can be rationalized by increased exposure and pK_a decrease.

Fig. 1. Reaction between FA-free or FA-bound HSA-SH and DTNB. (A) Reduced 5/1 palmitic acid-HSA (15 μM , 0.7 SH/HSA) was mixed with increasing concentrations of DTNB (75–600 μM) in phosphate buffer (100 mM, 0.1 mM DTPA, pH 7.4) using a stopped flow accessory and the absorbance at 412 nm was recorded at 25 $^{\circ}\text{C}$. Data (gray traces) were fitted to an exponential function (black traces). (B) Second-order rate constants of the reactions of FA-free HSA (black squares) and 5/1 palmitic acid-HSA (gray circles). Data represent the average of duplicates. Inset: Amplitudes obtained from the exponential fittings.

Fig. 2.

Reaction of FA-free or FA-bound HSA-SH with hydrogen peroxide. (A) Calibration curve for the hydrogen peroxide sensor obtained by the sequential additions of known amounts of H_2O_2 (arrows) to buffer MES (0.1 M), Tris (0.052 M) and ethanolamine (0.052 M) at 37 °C, pH 7.4. Inset: data fitted a straight line. (B) Reduced FA-free (118 μM , 0.85 SH/HSA) or FA-bound (144 μM , 0.7 SH/HSA) HSA were added to hydrogen peroxide (100 μM) under the same conditions as in (A).

Fig. 3. Reaction of FA-free or FA-bound HSA-SH with peroxynitrite. Increasing concentrations of FA-free (black squares, 101–286 μM, 0.70 SH/HSA) or FA-bound (gray circles, 104–278 μM, 0.74 SH/HSA) reduced HSA were mixed with peroxynitrite (15 μM) in phosphate buffer (100 mM, 0.1 mM DTPA, pH 7.4). The absorbance at 302 nm was recorded for 10 s at 37 °C and the observed rate constants (k_{obs}) were used to determine the second-order rate constants.

Fig. 4. pH dependencies of the reactions of FA-free or FA-bound HSA-SH with hydrogen peroxide and DTNB. (A) Reaction between HSA-SH and hydrogen peroxide. Reduced FA-free (black squares, 118 μM , 0.85 SH/HSA) or FA-bound (gray circles, 144 μM , 0.7 SH/HSA) HSA were added to hydrogen peroxide (100 μM) in buffer MES (0.1 M), Tris (0.052 M) and ethanolamine (0.052 M) of different pHs at 37 °C. The reaction was followed with a hydrogen peroxide electrode. (B) Reaction between HSA-SH and DTNB. Reduced FA-free (black squares, 24 μM , 0.83 SH/HSA) or FA-bound (gray circles, 15 μM , 0.69 SH/HSA) HSA were mixed with DTNB (100 μM or 50 μM , respectively) in the same buffer at 25 °C. The initial rate of the reaction was determined from the linear increment of absorbance at 412 nm.

Fig. 5. Reaction between FA-free or FA-bound HSA-SOH and TNB. (A) Reduced FA-free (0.25 mM, 0.85 SH/HSA) or FA-bound (0.25 mM, 0.72 SH/HSA) HSA were oxidized with hydrogen peroxide (2 mM, 5 min, 37 °C) and the reactions were stopped with catalase. Aliquots (25 μM) were incubated with TNB (70 μM) and the decrease in TNB concentration was recorded at 412 nm, 25 °C, pH 7.4. The black traces represent the best fits to exponential plus straight line functions. (B) The second-order rate constants were determined from the slopes of k_{obs} versus [TNB] for FA-free (black squares) and FA-bound (gray circles) HSA. Inset: Amplitudes of the exponential fittings against the TNB concentration. The lines indicate the mean values.

Fig. 6.

Reaction of FA-free or FA-bound HSA-SH and HSA-SOH with hydrogen peroxide. Reduced FA-free or FA-bound HSA (~0.25 mM, 0.80 SH/HSA) were incubated with hydrogen peroxide (2 mM) at 37 °C. At increasing times, aliquots (50 μM FA-free, black squares, or 25 μM FA-bound, gray circles) were mixed with catalase and TNB (70 μM or 35 μM, respectively). The initial rate of TNB decay at 25 °C was plotted against the time of incubation with H₂O₂. The solid lines represent the best fits to biexponential functions.

Fig. 7.

Competition between TNB and biologically relevant thiols for reaction with HSA-SOH. (A) Aliquots (25 μM) of oxidized (2 mM H_2O_2 , 5 min, 37 $^\circ\text{C}$) FA-bound HSA were mixed with TNB (35 μM) at 25 $^\circ\text{C}$ in the absence or presence of cysteine (406 μM), glutathione (3.0 mM), homocysteine (524 μM), or cysteinylglycine (140 μM) using a stopped flow accessory. The absorbance at 412 nm was recorded for 10 min. The gray lines represent the best fits to exponential plus straight line functions. (B) k_{obs} and amplitudes (Inset) for the reaction of FA-free (black squares) and FA-bound (gray circles) HSA-SOH with cysteinylglycine.

Fig. 8.

Binding of *cis*-parinaric acid to HSA-SH and HSA-SO₂H. Increasing concentrations of purified HSA, either reduced (black squares) or oxidized to sulfinic acid (gray triangles, 10 mM H₂O₂, 10 min, 37 °C) were incubated with *cis*-parinaric acid (2 μM, 10 min, 25 °C) and the fluorescence at 320/420 nm was measured at 25 °C during 10 s. Data are the average and standard deviation (n = 4). The lines represent the best fits to five consecutive equilibria.

Inset: Data at low HSA concentrations.

Fig. 9. Surface exposure (left panels) and amino acid microenvironment (right panels) of Cys34 in the absence and presence of FA. (A) FA-free HSA, PDB ID 1AO6. (B) HSA bound to stearic acid, PDB ID 1E7I. The amino acid residues are shown according to the element (C: violet, O: red, N: blue, S: yellow). Distances are expressed in angstroms. The figures were generated using PyMOL v0.99.