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# The thiol pool in human plasma: The central contribution of albumin to redox processes

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## Abstract

The plasma compartment has particular features regarding the nature and concentration of low and high molecular weight thiols and oxidized derivatives. Plasma is relatively poor in thiol-based antioxidants; thiols are in lower concentrations than in cells and mostly oxidized. The different thiol-disulfide pairs are not in equilibrium and the steady-state concentrations of total thiols as well as reduced versus oxidized ratios are maintained by kinetic barriers, including the rates of reactions and transport processes. The single thiol of human serum albumin (HSA-SH) is the most abundant plasma thiol. It is an important target for oxidants and electrophiles due to its reactivity with a wide variety of species and its relatively high concentration. A relatively stable sulfenic ($HSA\text{-}SO_3H$) acid can be formed in albumin exposed to oxidants. Plasma increases in mixed disulfides (HSA-SSR) or in sulfinic ($HSA\text{-}SO_2H$) and sulfonic ($HSA\text{-}SO_3H$) acids are associated with different pathologies and may constitute biomarkers of the antioxidant role of the albumin thiol. In this work we provide a critical review of the plasma thiol pool with a focus on human serum albumin.

### Keywords

Thiols; Albumin; Sulfenic acid; Mixed disulfides; Plasma; Oxidants; Free Radicals

## Introduction

In biological systems, thiols are found in cysteine and derived molecules of low and high molecular weight. Thiols are good reductants and good nucleophiles; they can react by one- and two-electron mechanisms and they are susceptible to reversible and irreversible modifications. In most proteins, cysteine accounts for less than 3% of the amino acid composition. However, its chemical versatility allows this residue to participate in several



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processes such as catalysis, signaling, antioxidant defense, metal complexing, and structural stabilization.

The plasma compartment is characterized by having relatively low concentrations of thiols and by the presence of human serum albumin (HSA) as the most abundant one. In the first part of this review we will discuss the thiol pool of plasma, and in the second part we will focus on HSA and its thiol (HSA-SH).

## Redox versatility of thiols

Thiols (RSH) can undergo oxidation processes to yield a wide range of products (Fig. 1). They can react with disulfides (RSSR) through reversible thiol-disulfide exchange reactions (pathway a in Fig. 1). These reactions can occur alone at appreciable rates or they can be catalyzed by enzymes of the thioredoxin family. Thiols can react with two-electron oxidants such as hydroperoxides (POOH) or hypohalous acids (i.e., HOCl) yielding sulfenic acids (RSOH) (pathway b in Fig. 1). Sulfenic acids are typically unstable and decay usually by reaction with another thiol, yielding disulfides. Thiols can also be oxidized by one electron to form highly reactive thiyl radicals ($RS^{\cdot}$) (pathway c in Fig. 1). Thiyl radicals can react with themselves forming disulfides, or with thiols forming disulfide radical anion ($RSSR^{\cdot-}$). These $RSSR^{\cdot-}$ radicals can reduce oxygen to superoxide radical ($O_2^{\cdot-}$). Alternatively, thiyl radicals can react with oxygen yielding peroxyl radicals ($RSOO^{\cdot}$) and other secondary radicals, which can yield oxyacids such as sulfenic, sulfinic ($RSO_2H$), and sulfonic ($RSO_3H$) acids.

As shown in Fig. 1, thiol oxidation converges on several products. Some of these products, like disulfides, can revert to thiols with suitable reductants. Other products such as sulfinic and sulfonic acids constitute typically final products [1–4].

## The reacting species is the thiolate

For most chemical and enzymatic reactions of thiols, reactivity involves the nucleophilic attack of the ionized thiolate ($RS^-$) on an electrophile. Thus, the observed rate constant for a certain reaction increases as the pH alkalinizes because more thiolate is available. In turn, if we compare different compounds, we can see that the availability of thiolate at a fixed pH depends on the p$K_a$ of the corresponding thiol; as the p$K_a$ of the thiol decreases, more thiol is ionized to thiolate.

The fact that thiolates and not thiols are the reactive species has led to the often-quoted misconception that reactive cysteines have low p$K_a$s. Thiols with low p$K_a$s have the advantage of higher availability of the thiolate at neutral pH. However, the intrinsic reactivity of the thiolate is diminished. Since nucleophilicity depends on the electron density of the reacting atom, it actually increases with basicity. For a certain reaction, the intrinsic reactivity of different thiolates, as measured by the pH-independent rate constant if all the thiol were ionized to thiolate, increases as the p$K_a$ increases. Within proteins, thiol p$K_a$ and nucleophilicity toward specific substrates can be modulated independently by the protein environment, giving rise to proteins with extraordinary thiol reactivity, such as the peroxiredoxins (for review see [5]).

## In plasma, total thiols are at a lower concentration than in cells and the predominant thiol is HSA

In cells, thiols are present at millimolar concentrations and highly reduced. The total glutathione concentration is 2–17 mM [6] and the percentage of reduced glutathione is 91%

for whole cells [7]. Specifically in the cytosolic compartment, glutathione is even more reduced, with recent evaluations yielding values of~99.97% [8,9]. Protein thiols are more abundant than glutathione (10–50 mM) [6]. They are~90% reduced and they represent ~70% of the total pool of reduced thiols [7]. Two important thiol/disulfide networks are represented in cells by glutathione, glutaredoxin, and glutathione reductase, and by thioredoxin and thioredoxin reductase. Intracellular thiols are ultimately kept reduced mainly by the action of NADPH.

In contrast, in the extracellular environment, and particularly in the plasma compartment, thiols are more oxidized and at much lower concentrations. As shown in Table 1, total reduced thiols in plasma add up to ~0.4–0.6 mM. Within this group, low molecular weight reduced thiols are represented by cysteine, cysteinylglycine, glutathione, homocysteine, and γ-glutamylcysteine, which together constitute only 12–20 μM. Remarkably, total glutathione is ~6 μM and~55% reduced. For the other low molecular weight thiols, the percentage reduced with respect to total is ~4% for cysteine, 9% for cysteinylglycine, 3% for homocysteine, and 1% for γ-glutamylcysteine. The most abundant thiol in plasma is HSA (~0.6 mM) and is mostly reduced (~75%).

Regarding the plasma thiol pool, homocysteine deserves special mention because increased concentrations are associated with cardiovascular disease and neural tube defects [10,11]. Similar to other low molecular weight thiols, homocysteine can form disulfide bonds. In addition, homocysteine thiolactone, the cyclic thioester derivative, can be formed and modify lysine residues leading to N-homocysteinylation of proteins [12].

Hydrogen sulfide ($H_2S/HS^-$), which can be considered the smallest member of the thiol family, also deserves special mention since it has been receiving increased attention in the last 15 years. Although hydrogen sulfide has been traditionally considered a toxic molecule, this vision is changing after it was recognized that not only can it be synthesized in mammals but also it can exert physiological effects with potential health benefits. Hydrogen sulfide is synthesized mainly as a by-product of the transsulfuration enzymes that catalize the formation of cysteine from methionine and is catabolized principally in the mitochondria (for review see [13]). Its intrinsic reactivity appears comparable to that of low molecular thiols [14]. The measurements of the concentrations of hydrogen sulfide that can be found in different tissues have been decreasing throughout the years, and recent estimates in blood yield values lower than micromolar [15].

## The different thiol-disulfide pairs are not in equilibrium with each other in plasma

If the different thiols and disulfides were in equilibrium in the circulation with regard to the thiol disulfide exchange reactions, the concentration quotients would equal the value of the equilibrium constants. For example, the exchange between cysteine and glutathione is represented by (actually, the reacting species are the thiolates)

$$\mathrm{CysSH} + \mathrm{GSSG} \rightleftharpoons \mathrm{CysSSG} + \mathrm{GSH} \quad (1)$$

$$\mathrm{CysSH} + \mathrm{CysSSG} \rightleftharpoons \mathrm{CysSSCys} + \mathrm{GSH}. \quad (2)$$

The overall reaction is described by

$$2\mathrm{CysSH}+\mathrm{GSSG} \rightleftharpoons \mathrm{CysSSCys}+2\mathrm{GSH}. \quad (3)$$

The corresponding equilibrium constant is defined by

$$K_{\mathrm{eq}}=\frac{[\mathrm{CysSSCys}]\,[\mathrm{GSH}]^2}{[\mathrm{CysSH}]^2[\mathrm{GSSG}]}. \quad (4)$$

The reported value for this equilibrium constant is 0.265 at neutral pH [16]. In contrast, the concentration quotient according to Eq. (4), determined using the values in Table 1, is ~6. In the case of homocysteine instead of cysteine, the equilibrium constant is 0.607 at neutral pH [16], while the concentration quotient is ~200 (Table 1). Clearly, the low molecular weight thiol pairs are not in equilibrium.

The thiol-disulfide exchange reactions that involve HSA are difficult to evaluate because the equilibrium constants are not known, neither are the rate constants of the corresponding reactions. The exchange between HSA-SH and cysteine disulfide is represented by

$$\mathrm{HSA}-\mathrm{SH}+\mathrm{CysSSCys} \rightleftharpoons \mathrm{HSA}-\mathrm{SSCys}+\mathrm{CysSH}. \quad (5)$$

It is reasonable to assume that the equilibrium constant of this reaction is~1 at neutral pH, based on the values of the corresponding equilibrium constants for low molecular weight thiols [16] and on the values calculated from equations relating the equilibrium constant to the pH and the p$K_a$s [17–19]. However, the concentration quotient is 0.07 (Table 1). If instead of cysteine disulfide we consider cysteinylglycine, homocysteine, or glutathione disulfide, the concentration quotients are 0.02, 0.005, and 0.01, again suggesting that the thiol-disulfide pairs are far from equilibrium.

## Possible kinetic barriers that keep plasma thiol and disulfide concentrations away from equilibrium

Since thiols and disulfides are not in equilibrium in plasma, it is clear that kinetic barriers exist. The steady-state concentrations are the result of several processes occurring simultaneously that affect not only the total concentrations but also the ratios of oxidized versus reduced thiols. Although qualitative information about these processes exists, precise quantitative information is mostly lacking. The plasma concentrations of thiols and oxidized derivatives are influenced by: (i) the rates of the thiol-disulfide exchange reactions; (ii) the rates of thiol oxidation by reactive oxygen species and possible repair processes; (iii) the rates of enzymatic extracellular degradation of glutathione; (iv) the rates of transport between the plasma compartment and cells, particularly erythrocytes and endothelial cells; (v) rates of liver release of thiol-containing molecules such as HSA and glutathione and, to a minor extent, rates of renal excretion; and (vi) intracellular metabolism.

It is often oversimplified that in extracellular milieus cysteine is oxidized to its disulfide quickly, invoking the mechanisms depicted in Fig. 1. In the test tube, cysteine and cysteinylglycine autooxidize faster than glutathione and homocysteine, probably because of their lower p$K_a$ [20,21]. Contrarily, in plasma, the disappearance of reduced cysteine and cysteinylglycine is slower than that of glutathione and homocysteine [22]. This highlights the contribution of different mechanisms to the maintenance of thiol steady states.

To illustrate on the complexities involved, it is useful to follow the fate of a reduced thiol that enters the intravascular space. Intravenous administration of reduced glutathione leads

first to an increase in plasma concentration that is followed by elimination in a timescale of minutes. Elimination is paralleled by an increase in plasma cysteine and its entrance into cells. The formation of cysteine is influenced by the hydrolysis of glutathione by extracellular transpeptidase and dipeptidase activities [23,24].

Intravenous administration of reduced homocysteine leads to a peak in plasma concentration in ~1 min, that precedes peaks in reduced cysteine and cysteinylglycine, maximal after ~4 min. Low molecular weight disulfides of homocysteine lag behind, while those of cysteine and cysteinylglycine do not change. Protein-bound homocysteine shows the slowest increase in concentration [25]. These results are consistent with reduced homo-cysteine reacting first with HSA-SSCys to release HSA-SH and CysSSHcy, followed by further reaction of HSA-SH with CysSSHcy, forming HSA-SSHcy and cysteine. This mechanism has been confirmed by *in vitro* experiments [26] and shows that HSA is an active player within the plasma thiol pool.

The thiol redox status in plasma can be altered pharmacologically. For example, *N*-acetylcysteine has been widely used for the treatment of paracetamol (acetaminophen) poisoning, among other conditions. When administered intravenously, *N*-acetylcysteine plasmatic concentrations transiently increase, mainly altering the pool of protein-bound thiols through a thiol-disulfide exchange reaction. This leads to the formation of mixed low molecular weight disulfides with increased renal clearance. Furthermore, *N*-acetylcysteine can be converted to cysteine which is then incorporated into glutathione in the liver helping to replenish its levels [27,28].

## The extracellular low molecular weight thiol pool is not an inert bystander but actively affects cell processes

The extracellular concentrations of different thiols and oxidized derivatives are tightly regulated, and its alterations are associated with cellular responses. Cells in culture respond to increased ratios of cysteine disulfide to cysteine in the extracellular medium through downstream events that impact on proliferation, apoptosis, and proinflammatory signaling (for review see [29]). In line with this concept, the *in vivo* concentrations of plasma low molecular weight thiols and disulfides have been found altered in association with conditions such as aging, smoking, obesity, and alcoholism among others [30]. The molecular mechanisms linking extra- and intracellular phenomena probably involve the oxidation of thiols in membrane proteins that trigger signaling events.

Another important point of interplay has to do with the transport of cysteine disulfide into cells. Cysteine disulfide can be reduced intracellularly, leading to increased glutathione synthesis and excretion of surplus cysteine. The uptake of cysteine disulfide is closely related to the transport of glutamate. This provides a link to glutamate toxicity in the brain [31,32].

## Antioxidants are scarce in plasma and HSA is in the spotlight as the predominant thiol

Plasma low molecular weight antioxidants include reduced thiols, ascorbate, urate, among others (Tables 1 and 2). Reduced low molecular weight thiols are in very low concentrations, adding up to a total of 12–20 μM.

Despite the plasma proteome comprising more than 3000 components, proteins with potential antioxidant function are also scarce in this compartment. Among the 150 most

abundant polypeptides, only two possess antioxidant capacity: human serum albumin and glutathione peroxidase 3 [33] (Tables 1 and 2).

On the other hand, the erythrocyte is able to play a relevant role as oxidant scrubber. Contrary to plasma, the erythrocyte is rich in antioxidant defenses. It can be estimated that, since the mean distance between two erythrocytes is ~3 μm, an oxidant molecule that diffuses in a typical way ($10^{-5}$ $cm^2/s$) will reach the erythrocyte in a few milliseconds. Thus, in those cases where cell membranes do not constitute a barrier, oxidants will likely encounter the antioxidant battery of the erythrocyte.

## HSA is a monomeric, three-domain, allosteric protein with one free cysteine

HSA is a monomeric, nonglycosylated, three-domain protein. It is present in plasma at a concentration of ~43 g/L (~0.6 mM), constituting about 60% of total plasma proteins. It has 585 amino acids (66438 Da) and 17 disulfide bridges leaving only one free cysteine, Cys34. At pH 7.4, HSA possesses 215 ions with a net charge of −19 conferring HSA a high solubility [34].

The crystal structure of HSA has already been solved and it shows that it is a heart-shaped protein with 67% α-helixes and no β-sheets [35] (Fig. 2). Cys34 is located in the surface of domain I, with the sulfur oriented toward the interior of the protein. It is surrounded by the side chains of Pro35, Val77, Asp38, His39, and Tyr84. The last three residues are of relevance due to the presence of ionizable groups [35,36].

The HSA molecule undergoes several well-characterized reversible conformational changes, usually under nonphysiological pH conditions [34]. Ligand binding also triggers conformational changes in the HSA molecule. Fatty acids induce global and local changes. Globally, fatty acids promote relative rotations of the three domains [37,38]. Local changes are more subtle but specially relevant at the level of Cys34.

## HSA is synthesized in the liver and present in intravascular and extravascular compartments

HSA synthesis takes place in the hepatocytes accounting for 10% of total protein synthesis in the liver [39]. HSA follows the rule “one gene–one protein” and as a monomeric protein no chain assembly is needed. When isolated from plasma, HSA contains 585 amino acids; however, when first translated from RNA it contains a prepropeptide at the N-terminus which is removed in the hepatocyte before secretion. Disulfide formation starts before the chain is completed and proceeds in the N-terminal to C-terminal direction. All of the 17 disulfide bonds are formed within 0.5 min of release of the completed chain from the parent polysome. It is not clear why Cys34 does not participate in disulfide bonding [34].

Once synthesized HSA is immediately secreted, there is no HSA storage in the hepatocyte. Only a small percentage (<2%) is found in the hepatocyte because of the 30 min trip before secretion. The half-life of an HSA molecule is ~27 days. Once secreted only 40% (~120 g) of HSA remains in the intravascular compartment while the remaining 60% (~160 g) is found in the extravascular compartment, mainly in skin and muscle [34]. An HSA molecule leaves the circulation through the lymphatic system each 22–24 h by different mechanisms depending on the tissue involved. About 50% of HSA transport from the circulation occurs in liver, bone marrow, pancreas, small intestine, and adrenal glands where there is unimpeded passage of plasma (fenestrated or sinusoidal endothelium). In the remainder of the body (heart, lung, brain, fat, skeletal muscle, diaphragm, mesentery, duodenal musculature) there is a continuous endothelium and HSA leaves the circulation by an active

transcytotic mechanism via vesicles. Albondin (gp60), a glycosylated receptor, specifically binds native HSA providing a mechanism for the facilitated delivery of the protein and its bound ligands to the interstitium [34,40–43].

All tissues degrade HSA, but muscle, liver, and kidney are the main contributors to HSA catabolism (40–60% of the HSA dose) [44,45]. Modified or conformationally altered HSA does not bind to albondin but to a different set of receptors namely gp18 and gp30. They mediate nonnative HSA binding, caveolae-dependent endocytosis and degradation to free amino acids in the lysosome. They are distributed ubiquitously regardless of both the type of endothelium and the local mechanism of transendothelial transport of HSA and they are present in lysates of fibroblasts and smooth muscle cells as well [42,46,47]. It has also been reported that oxidative modifications that lead to changes in the hydrophobicity and net charge increase the rate of elimination of HSA [48].

## HSA has several functions including the binding of exogenous and endogenous compounds

HSA plays several roles in the circulation. HSA is responsible for 80% of the oncotic pressure in plasma. Due to the 19 negative charge of HSA at pH 7.4, it constitutes the principal macromolecular anion in plasma [34].

The multidomain structure allows HSA to bind and transport a wide variety of endogenous and exogenous ligands. Long and medium chain fatty acids constitute some of the most important endogenous ligands. These relatively insoluble and metabolically active molecules are transported in HSA from the intestine to the liver, from the liver to muscle, and from and to the adipose tissue. In physiological conditions HSA binds 0.3–1 fatty acids per molecule [49]. This ratio is increased during intense exercise, adrenergic stimulation, and in pathological conditions like diabetes mellitus [34]. In fact, HSA is capable of binding up to 9 fatty acids per molecule. The nine binding sites are distributed all along the protein in an asymmetric way and they present different affinities, although they all have an hydrophobic pocket that interacts with the hydrocarbon chain of the fatty acid and a basic or polar residue that interacts with the carboxylate [50,51]. HSA also binds metals, vitamins, hormones, bilirubin, biliar salts, hemin, and exogenous drugs. It has also been reported that HSA is capable of binding peptides and proteins. These associations are of special relevance in plasma proteomic studies where usually removal of HSA is the first step in sample processing and have led to a new term: “the albuminome” [52].

HSA is proposed to exert an antioxidant function. This derives in part from its bound ligands. HSA binds bilirubin, which has been proposed as an antioxidant due to its capacity to scavenge lipid peroxyl radicals ($ROO^{\cdot}$) [53]. Furthermore, bilirubin bound to HSA is not able to cross the hematoencephalic barrier, thus preventing neurotoxic consequences [54]. In addition, HSA modulates the outcome of Fenton chemistry involving redox active metals. For example, the binding of copper ions to a high affinity site located in the N-terminus of HSA partially inhibits oxidation processes [55,56]. The net effect of HSA on metal catalyzed oxidation depends on the nature of the metal ion (e.g., copper or iron) in relation to its binding sites, on the metal/HSA ratios, and on whether oxidation of HSA itself or of an exogenous molecule is measured, since residues in HSA are able to react with the oxidants formed [55–58]. Indeed, HSA constitutes an important target for reactive species and, as described later, Cys34 critical for this property of paramount importance in its proposed antioxidant role.

HSA therapy has been used for more than 50 years for the treatment of several conditions related to hypovolemia. Still, the use of HSA solutions instead of alternative fluids is

controversial [59–61]. HSA is also of pharmaceutical interest as a drug carrier [62], and several HSA-based drugs have received market approval or are in clinical development [63]. The availability of recombinant HSA has been essential for broadening the pharmaceutical applications of the protein [64–66].

## HSA is heterogeneous regarding the oxidation state of Cys34 *in vitro* and *in vivo*

The presence of a free thiol in HSA, Cys34, gives rise to heterogeneity. In plasma, HSA is found as a mixture of mercaptalbumin with the thiol in the reduced form (HSA-SH) and non-mercaptalbumin with the thiol modified (Table 1). In healthy adults, about 75% of HSA is found as mercaptalbumin. The nonmercaptalbumin fraction consists mainly of mixed disulfides of HSA with low molecular weight thiols [67,68]. About 1–2% of total HSA is found with the thiol oxidized to higher oxidation states such as sulfinic acid (HSA-$SO_2H$) or sulfonic acid (HSA-$SO_3H$) [69–73]. The pool of HSA mixed disulfides is roughly equivalent to that of protein mixed disulfides, since protein-bound thiols are mostly (70–90%) linked to HSA and the binding to other proteins such as globulins is minor [74]. Within the pool of HSA mixed disulfides, those with cysteine are by far the most abundant (~86%), followed by cysteinylglycine (~8%), homocysteine (~5%), and glutathione (~1%). In addition, it is worth noting that the low molecular weight thiols mostly circulate bound to HSA (Table 1).

The HSA thiol can also be found nitrosylated (*S*-nitrosoalbumin, HSA-SNO). This species, which has been detected at concentrations of <0.2 μM [75,76] can act as a nitric oxide reserve and take part in transnitrosation reactions. In addition, Cys34 can be found forming carbonylated adducts due to a Michael addition to α-β unsaturated aldehydes such as 4-hydroxy-*trans*-2-nonenal [77]. This reaction has also been reported with nitrated fatty acids, leading to the formation of a thiol-nitroalkene adduct [78].

## The HSA thiol reacts with a wide variety of oxidant species

Honoring its thiol nature, HSA-SH can react in the test tube with most typical radical and nonradical oxidants. The occurrence of these reactions *in vivo* is evidenced by the detection of thiol oxidized isoforms of albumin in the circulation, that increase in conditions related to oxidative stress. The exact nature of the oxidant species involved cannot be elucidated from product characterization studies, since the final products of the reactions are expected to overlap. So what oxidants could actually be oxidizing HSA-SH in plasma? To critically analyze this it is key to keep in mind that oxidation will depend on kinetic factors of rate constant and concentration of both HSA-SH and potentially competing targets, together with diffusion and compartmentalization aspects.

As examples of the considerations involved, we can consider the cases of hydrogen peroxide and peroxynitrite. Hydrogen peroxide reacts with HSA-SH with a rate constant of 2.3±0.7 $M^{-1}$ $s^{-1}$ (37 °C, pH 7.4) [79–81]. One possible competitor for HSA-SH could be glutathione peroxidase 3, which is present at a concentration of 0.5–0.8 μM (Table 2) and reacts at 1.8 $\times 10^8$ $M^{-1}$ $s^{-1}$ [82]. However, it is not clear whether this enzyme is active due to the paucity of reducing substrates. Nevertheless, hydrogen peroxide will probably reach the erythrocyte and be rapidly consumed by peroxiredoxin 2 [83] before encountering an HSA molecule. In the case of peroxynitrite, carbon dioxide will outcompete HSA-SH. The reaction with carbon dioxide occurs at $4.6 \times 10^4$ $M^{-1}$ $s^{-1}$ (37 °C, pH 7.4) [84] and the concentration of $CO_2$ is 1.3 mM, giving a product of rate constant ×concentration of 60 $s^{-1}$, while the reaction with HSA-SH occurs at $3.8 \times 10^3$ $M^{-1}$ $s^{-1}$ (37 °C, pH 7.4) [85] and a concentration of 0.42 mM, giving a product of 2 $s^{-1}$. However, the reaction of peroxynitrite with carbon

dioxide leads to the formation of nitrogen dioxide ($^{\cdot}NO_2$) and carbonate ($CO_3^{\cdot-}$) radicals. These will react rather unspecifically with low molecular weight targets and different protein residues including HSA-SH [86].

These concepts are supported by an experiment where the oxidation state of HSA-SH was evaluated in plasma and blood after exposure to hydrogen peroxide and peroxynitrite. When hydrogen peroxide was used, the erythrocytes highly prevented HSA-SH oxidation. In the case of peroxynitrite, the inhibition was significantly lower since peroxynitrite mainly reacted with carbon dioxide forming nitrogen dioxide and carbonate radicals which in turn reacted with HSA-SH [87].

## Cys34 p$K_a$: A long-standing controversy

The HSA-SH acidity constant (p$K_a$) has been reported as <7 [36] or even <5 [88]. Other reports have shown values of about 8 [85,80,89]. Three aspects may contribute to the complexities regarding the determination of this p$K_a$ and the diversity of results obtained. First, according to NMR experiments, the thiol exists in two conformations and the reduced thiol predominates in a buried conformation that shifts to an exposed one upon chemical modification [90,91]. Second, HSA suffers conformational changes with pH that also affect the thiol reactivity. For example, the reaction with the disulfide 2,2′-dithiodipyridine showed an increase at pH ~7 probably due to thiol deprotonation and an increase at pH<5 [92] probably due to a pH-dependent conformational transition [34]. Third, the p$K_a$ can be modulated by ligands [80], so the source of albumin for the experiments is critical.

In our view, the p$K_a$ of ligand-free HSA-SH is ~8. From the pH dependency of the reaction with hydrogen peroxide, we have determined a value of 7.9±0.1 [80]. We consider this to be a good estimate because hydrogen peroxide is a small and neutral molecule that reacts specifically with the thiol. Similar values can be obtained using peroxynitrous acid [85] or the disulfide 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [80,89] as reactants. The p$K_a$ of ~8 was confirmed by UV-Vis titration and FTIR vibrational spectroscopy (unpublished results).

If we compare the value of the p$K_a$ of HSA-SH (8) with that of a typical peptide (9.1 [93]), we can conclude that the thiolate in HSA is moderately stabilized by the environment. Stabilization is probably mediated by Tyr84, located within hydrogen bonding distance (3.1±0.2 Å according to the crystal structures reported with ligand-free HSA, chains A and B of 1AO6 and 1BM0), and by His39. Mutations of these residues lead to higher p$K_a$s [36]. The location in the N-terminus of an α-helix probably contributes to thiolate stabilization.

## The reactivity of HSA-SH is restricted by the protein environment

When we compare HSA-SH against low molecular weight thiols, its diminished reactivity becomes evident. With hydrogen peroxide, a small oxidant, the pH-independent rate constant (when all the thiol is in the thiolate form) for HSA is fourfold lower (5.3±0.2 $M^{-1}$ $s^{-1}$) [80] than those for low molecular weight thiolates of comparable p$K_a$ (18–26 $M^{-1}$ $s^{-1}$) [94]. With peroxynitrous acid, the HSA thiolate reacts at 7.9 ×$10^4$ $M^{-1}$ $s^{-1}$ [85] while low molecular weight thiolates react at 1.3 ×$10^5$–2 ×$10^5$ $M^{-1}$ $s^{-1}$ [95]. Differences are more pronounced when we compare the reactivities with DTNB, a larger molecule. The HSA thiolate reacts at 40 $M^{-1}$ $s^{-1}$ [80] while low molecular weight thiols with p$K_a$s between 7.3 and 8.6, at 3 ×$10^4$–1.5 ×$10^5$ $M^{-1}$ $s^{-1}$ [96,97], three orders of magnitude faster.

Thus, the reactivity of HSA-SH, particularly toward voluminous targets, is restricted by steric constraints that probably stem from its location in a crevice and its limited solvent exposure (Fig. 2). Indeed, from available structural data it can be calculated that the water-exposed surface of the sulfur atom of Cys34 is 0.7 $Å^2$ [87]. This does not imply an inability

of the oxidants to reach the thiolate, since these reactions are far from diffusion limited, but rather an impediment for adequate geometric alignment between reactants.

It is likely that the location of the thiol in a crevice also impacts on the paucity of formation of intermolecular disulfides (HSA-SS-HSA) or thiosulfinates (HSA-S(O)S-HSA), that have never been observed in our laboratory. It can be hypothesized that this represents an advantage because of the potentially deleterious effect of HSA dimer formation due to a decrease in the oncotic pressure.

## Ligand binding affects thiol reactivity

As noted before HSA plays a key role in fatty acid transport between tissues. Despite the fact that Cys34 is not directly located in a fatty acid binding site, its oxidizability is affected [55,98,99]. When we studied the effect of fatty acid binding on Cys34 reactivity we found that the stearic acid-HSA (5/1) complex reacted sixfold faster than fatty acid-free HSA at pH 7.4 with DTNB and twofold faster with hydrogen peroxide and peroxynitrite. We also observed a decrease in p$K_a$ by 0.5 pH units upon fatty acid binding, that can be explained in terms of a more polar microenvironment, in line with an increase in the surface exposure of the thiol. The lower p$K_a$ leads to a higher thiolate proportion at pH 7.4 (54 versus 24%). Thus, the increased reactivity of HSA-SH at neutral pH can be rationalized by both increased availability of the thiolate species along with decreased steric restrictions [80].

On the other hand, the effect of HSA-SH oxidation on the protein conformation is a less explored area. It has been reported that oxidation impairs protein ligand capacity and increases its susceptibility to proteolytic degradation. However, binding experiments with *cis*-parinaric acid (fluorescent conjugated polyunsaturated fatty acid, $C_{18}H_{28}O_2$) and reduced HSA (HSA-SH) or overoxidized HSA (HSA-$SO_2$H) showed no correlation between the thiol oxidation state and the binding capacity of the protein [80].

## A relatively stable sulfenic acid is formed in HSA

Sulfenic acid formation in HSA was observed more than 60 years ago [100] and confirmed with different analytical techniques [79,85,101–104]. However, for a comprehensive characterization of its reactivity and stability it was necessary to obtain quantitative data on its formation. We have developed a strategy for quantifying sulfenic acid (HSA-SOH) formed after exposure of HSA-SH to oxidants [81]. Thionitrobenzoate (TNB) reacts with HSA-SOH, yielding the mixed disulfide HSA-STNB leading to a decrease in the absorbance at 412 nm. Through careful kinetic analysis of the reaction we were able to measure the HSA-SOH concentration and thus opened the way to determining its rate constants with molecules of biological and analytical interest.

Once formed, HSA-SOH can follow one of three possible routes [81]. First, it can be further oxidized to sulfinic acid (HSA-$SO_2$H). The rate constant for hydrogen peroxide is 0.4 $M^{-1}$ $s^{-1}$ (pH 7.4, 37°C). The formation of HSA-$SO_2$H was confirmed by mass spectrometry with trypsinized and whole protein. Second, HSA-SOH can react with low molecular weight thiols, forming mixed disulfides. Through a competition approach we determined the rate constants for the reactions between HSA-SOH and plasma low molecular weight thiols. At pH 7.4 and 25 °C, HSA-SOH reacted with cysteine, cysteinylglycine, homocysteine, and glutathione with rate constants of 21.6, 55, 9.3, and 2.9 $M^{-1}$ $s^{-1}$, respectively. The reaction mechanism involved the nucleophilic attack of the thiolate on the sulfenic acid. Glutathione reacted more slowly than expected from its p$K_a$ probably due to its additional negative charge [87]. Last, HSA-SOH can decay spontaneously in solution with first-order rate constants at pH 7.4 of 1.7 $\times 10^{-3}$ $s^{-1}$ (37 °C) and 5.6 $\times 10^{-4}$ $s^{-1}$ (25 °C) to a yet uncharacterized product.

Taking into account the measured rate constants and plasma concentrations it is possible to conclude that the main routes for an *in vivo* formed HSA-SOH will be the reaction with low molecular weight thiols or the spontaneous decay. It can be estimated that the half-life for HSA-SOH in plasma will be ~4.5 min. This relatively short half-life in the context of the methodological complexities inherent to detecting sulfenic acids is probably the factor why HSA-SOH has not yet been detected in plasma samples.

When comparing with other proteins where sulfenic acid is formed, such as peroxiredoxins, HSA-SOH is much more stable. The main factors to account for this stability are the absence of neighbor thiols and the location in a crevice that impedes intermolecular reactions.

## Oxidized forms of HSA are increased in several pathophysiological conditions

Oxidative stress can be defined as an imbalance between prooxidants and antioxidants in favor of the former [105] or, from a mechanistic standpoint, as a disruption of redox signaling and control [106]. This condition is linked to a growing number of human diseases and pathophysiological processes. HSA-SH, as the most abundant thiol in plasma, is expected to be oxidatively modified in these situations.

The HSA redox state has been assessed in diverse conditions using mass spectrometry, chromatography, and other analytical techniques. These analyses have shown a decrease in reduced HSA which correlates with an increase in oxidized forms of the protein (Table 3). Depending on the study, both mixed disulfides and/or higher oxidation states (sulfinic and sulfonic acid) have been found increased.

*In vitro*, mixed HSA disulfides can revert to HSA-SH with suitable reductants including low molecular weight thiols, acting through thiol disulfide exchange reactions; little is known about reversal *in vivo.* In one study concerning strenuous exercise [107] where increases in plasma HSA mixed disulfides were measured, no recovery was observed 30 min postexercise and values returned to baseline by 30 h. This study sets a relatively slow timescale for reversal. Since the half-life of an HSA molecule is ~27 days, it is likely that mechanisms for reversal different from protein turnover exist, but enzymatic mechanisms have not been established, nor have the quantitative details regarding possible disulfide exchange processes. As for oxidation to sulfinic and sulfonic acids, these modifications are most likely irreversible.

The oxidative modifications of HSA are not present when it is secreted from the liver. While usually measured in plasma samples, they are not necessarily indicative of oxidation events occurring in that compartment, since a large percentage of HSA is located extravascularly. Whether the oxidation of HSA brings about physiological consequences remains to be established. So far, the oxidative modifications of HSA may constitute potential biomarkers of the implication of oxidative stress in pathophysiological processes and of the antioxidant role of HSA-SH.

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## Abbreviations

| | |
|---|---|
| **GSH** | reduced glutathione |
| **HSA** | human serum albumin |
| **HSA-SH** | thiol of human serum albumin |
| **HSA-SOH** | sulfenic acid in human serum albumin |
| **HSA-$SO_2H$** | sulfinic acid in human serum albumin |
| **HSA-$SO_3H$** | sulfonic acid in human serum albumin |
| **DTNB** | 5,5′-dithiobis(2-nitrobenzoic acid) |

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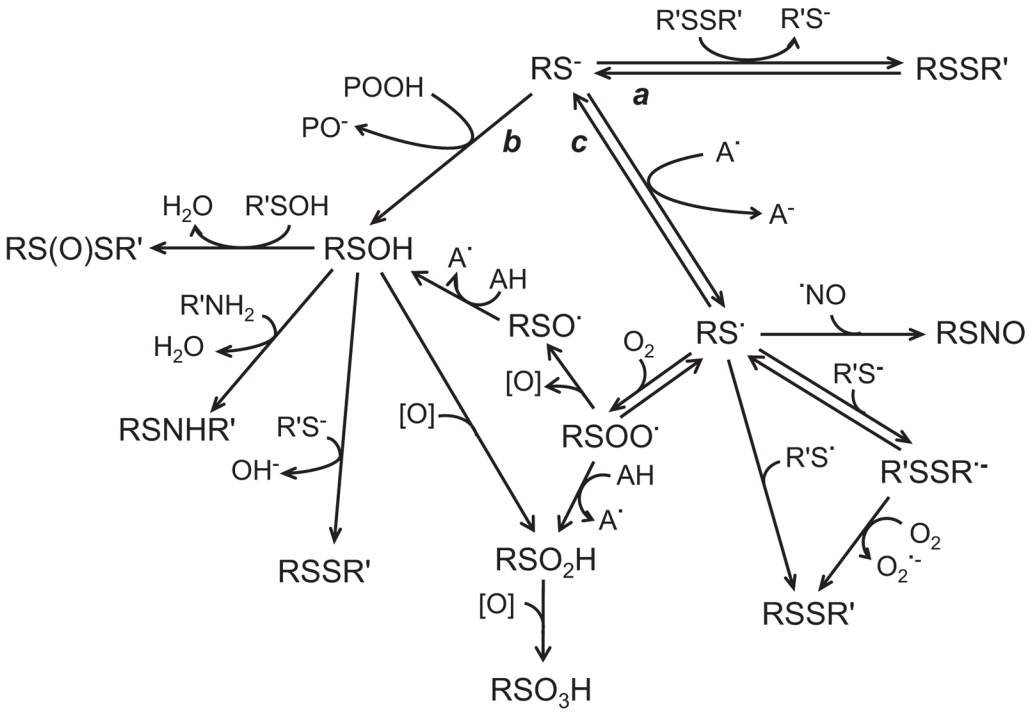


**Fig. 1.**

Pathways of thiol oxidation. (a) Thiol-disulfide exchange reaction. (b) Two-electron oxidation of a thiol, yielding a sulfenic acid (RSOH) and subsequent reactions. (c) One-electron oxidation of a thiol, yielding a thiyl radical (RS·) and subsequent reactions.

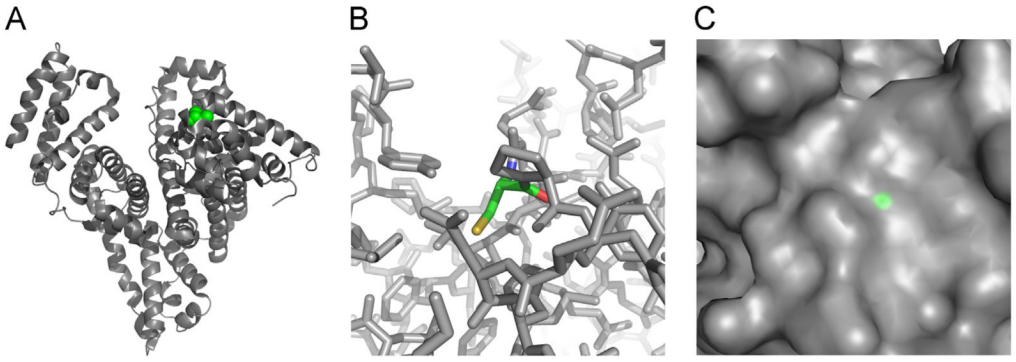


**Fig. 2.**

Three-dimensional structure of human serum albumin, local environment and surface exposure of Cys34. (A) Cys34 is shown in green. (B) Thiol microenvironment: C, green; O, red; N, blue; S, yellow. (C) Surface exposure of Cys34. Atomic coordinates were downloaded from Protein Data Bank, Accession Code 4EMX. The figures were prepared using PyMOL v0.99 (www.pymol.org).

## Table 1

Concentration of thiols and derived species in plasma from healthy adults.

| Species | Concentration (μM) | References |
|---|---|---|
| Total albumin | 527–783 | [34,7] |
| Protein thiols | 400–600 | [108,109] |
| Albumin thiol | 422±52 | [110] |
| Cysteine | | |
| Total[a] | 202–281 | [67,68,110–112] |
| Reduced | 8.3–10.7 | [67,68,108,110,112] |
| LMW disulfide | 41–63 | [67,68,108,110] |
| Protein mixed disulfide | 145–176 | [67,68,108,110,112] |
| Cysteinylglycine | | |
| Total[a] | 18.6–35.8 | [67,68,110,112] |
| Reduced | 2.0–2.9 | [67,68,108,110,112] |
| LMW disulfide | 4.4–6.8 | [67,68,108,110] |
| Protein mixed disulfide | 11–20 | [67,68,108,110,112] |
| Homocysteine | | |
| Total[a] | 6.5–11.9 | [67,68,110–112] |
| Reduced | 0.17–0.32 | [67,68,108,110,112] |
| LMW disulfide | 1.0–1.2 | [67,108,110] |
| Protein mixed disulfide | 7.3–10.4 | [67,68,108,110,112] |
| Thiolactone | $0–34.8 \times 10^{-3}$ | [113] |
| Glutathione | | |
| Total[a] | 4.9–7.3 | [67,68,110,112] |
| Reduced | 2.0–5.1 | [67,68,108,110,112] |
| LMW disulfide | 0.7–1.6 | [67,68,108,110] |
| Protein mixed disulfide | 0.7–1.9 | [67,68,108,110,112] |
| γ-Glutamylcysteine | | |
| Total | 3.1–5.4 | [67,111] |
| Reduced | 0.06 | [67] |
| Hydrogen sulfide | | |
| Total | $1 \times 10^{-4}$ | [15] |

[a]Total concentration =Reduced + 2 × LMW disulfide +Protein mixed disulfide.

## Table 2

Main plasma antioxidants.

| Species | Concentration (μM) | References |
|---|---|---|
| *Low molecular weight* | | |
| Reduced thiols | 12–20 | [67,68,108,112] |
| Ascorbic acid | 30–150 | [114,115] |
| Uric acid | 160–450 | [114,115] |
| α and γ-tocopherol[a] | 15–40 | [114,115] |
| Lycopene[a] | 0.5–1.0 | [114] |
| β-Carotene[a] | 0.3–0.6 | [114] |
| Ubiquinol 10[a] | 0.1–1.0 | [114] |
| Bilirubin[b] | 5–20 | [114] |
| *High molecular weight* | | |
| Albumin | 527–783 | [34,74] |
| Glutathione peroxidase 3[c] | 0.5–0.8 | [116] |
| Selenoprotein P | 0.1 | [117] |
| Thioredoxin | $0.07–1 \times 10^{-3}$ | [118] |
| Thioredoxin reductase | $3 \times 10^{-4}$ | [119] |
| Glutaredoxin | $1.1 \times 10^{-3}$ | [120] |

[a] Lipid-soluble antioxidants present in lipoproteins.

[b] Mainly bound to HSA.

[c] It is unknown if it is active due to the low concentration of its reductant substrates (GSH, Trx).

## Table 3

Oxidized HSA thiol in pathophysiological conditions and human diseases.

| Condition | Method | Observation/modification | References |
|---|---|---|---|
| Aging | Chromatography[a] | Decrease in the HSA-SH fraction | [121] |
| | Thiol measurement | Decrease in the SH/HSA ratio | [122] |
| | Thiol measurement with HPLC | Decrease in reduced protein thiols | [108] |
| Focal segmental glomerulosclerosis | Mass spectrometry (LC-ESI MS/MS for peptides, ESI MS for whole protein) [b] | Detection of sulfonic acid in HSA | [71,72] |
| Renal dysfunction | Chromatography[c] | Increase in the oxidized fractions with a decrease of renal function | [123] |
| Diabetes mellitus | Chromatography[c] | Increase in the oxidized fractions | [124] |
| Disorders of the temporomandibular joint | Chromatography[c] | In synovial fluid, increase in the oxidized fractions | [125] |
| Senile cataract | Chromatography[c] | In HSA from the aqueous humor, increase in the oxidized fractions | [126] |
| Invasive surgery and anesthesia | Chromatography[a] | Increase in the oxidized fractions | [127] |
| Liver disease (liver cirrhosis, acute-on chronic liver failure) | Chromatography[c] | Increase in the oxidized fractions | [128] |
| Strenous exercise | Chromatography[c] | Increase in the oxidized fractions | [129] |
| Intrauterine growth restriction | Mass spectrometry (LC-ESI-TOF of whole protein)[d] | High levels of cysteinylated maternal HSA | [130] |
| Hemodialysis | Chromatography[c] | Increase in the oxidized fractions | [131,132] |
| | SDS-PAGE and Western blot | Increase in HSA dimers | [133] |
| Neurodegenerative diseases | Chromatography[c] | Increase in mixed disulfides | [134] |
| Cardiovascular disease in peritoneal dialysis patients | Chromatography[c] | Decrease in the HSA-SH fraction | [135] |

[a] Anion exchange-hydrophobic interaction chromatography. Asahipak GS-520H column; eluent buffer 0.03 sodium phosphate, 0.3 M trisodium citrate, pH 6.86–6.80.

[b] LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry.

[c] Anion exchange-hydrophobic interaction chromatography. Shodex Asahipak ES-502N column; 0.4 M sodium sulfate, 0.05 M acetate, pH 4.85 as mobile phase and ethanol gradient (0 to 5–10%) for elution.

[d] LC, liquid chromatography; ESI, electrospray ionization; TOF, time of fiight.