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Review

Sulfenic acid—A key intermediate in albumin thiol oxidation☆

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ABSTRACT

The single thiol of human serum albumin (HSA-SH) is the predominant plasma thiol. Both circulating albumin and pharmaceutical preparations are heterogeneous regarding the thiol redox status, as revealed by anion-exchange–hydrophobic interaction chromatography. Sulfenic acid (HSA-SOH) is an intermediate in HSA-SH oxidation processes that was detected through different techniques including mass spectrometry. Recently, quantitative data led to the determination of rate constants. The preferred fate of HSA-SOH is the formation of mixed disulfides. Alternatively, HSA-SOH can be further oxidized to sulfinic and sulfonic acids. Oxidized forms increase under disease conditions, underscoring the importance of HSA-SH as a plasma scavenger of intravascular oxidants. We here provide a critical review of the oxidation of HSA-SH in the context of the intravascular compartment, with emphasis in the methodological approaches of mass spectrometry and chromatography for the analysis of albumin thiol redox states.

Contents

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1. Human serum albumin

1.1. Structure and metabolism

Human serum albumin (HSA, 66.5 kDa) is the most abundant protein in plasma (~43 g L^{-1}, 0.6 mM), constituting 60% of total protein [1]. Its non-glycosylated single chain of 585 amino acids contains 17 disulfide bridges and only one free cysteine, Cys34, which lies in a 9.5–10 Å crevice. This cysteine represents the largest fraction (>80%) of free thiols in plasma.

Within its monomeric heart-shaped structure, albumin contains 67% of alpha helixes and no beta sheets. It is modular, with three homologous domains connected by random coil, named I, II and III, each containing two subdomains, A and B, that probably originated from gene duplication (Fig. 1).

Albumin synthesis occurs predominantly in hepatocytes at a rate of ~10–15 g per day in healthy adults, accounting for at least 10% of liver protein synthesis [2]. There is no storage of newly synthesized albumin, only a small amount (<2%) in transit during its 30 min trip through the hepatocyte. The half-life of albumin is ~19 days. Approximately 40% is maintained within the plasma compartment while the remaining ~60% is transferred to the extravascular space at a rate of 4–5% per hour, returning to the intravascular compartment at an equivalent rate through the lymphatic system [1].

Albumin is degraded mostly in muscle, skin, liver and kidney. Degradation is enhanced if albumin is altered. In fact, chemically modified albumin (such as maleylated and formylated), denatured albumin generated under oxidative stress conditions, or albumin altered in specific amino acid residues, is preferentially recognized over native protein and taken up from circulating blood by several receptors which have widespread organ distribution, resulting in rapid internalization and degradation [3–6]. For example, in cultured rat liver endothelial cells, endocytosis mediated by cell-surface receptors gp18 and gp30 constitutes the primary pathway for lysosomal degradation of oxidative stress-denatured albumin [7].

1.2. Classical physiological functions

Albumin has a fundamental role in the maintenance of colloid osmotic pressure. With its ~19 negative charges at pH 7.4, it is the principal macromolecular anion in plasma and can also act as a pH buffer.

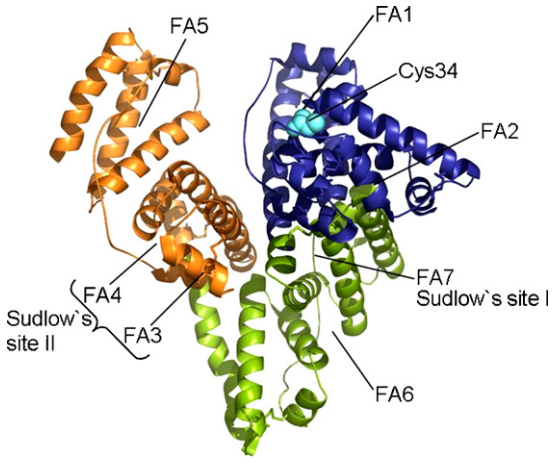

Fig. 1. Three dimensional structure of human serum albumin. Domains 1, 2 and 3 are shown in blue, green and orange, respectively. Cysteine 34 is shown in cyan. FA1–7 refer to fatty acid binding sites. Sudlow's sites refer to multi-specific high-affinity sites. Atomic coordinates were downloaded from the Protein Data Bank, accession code 1AO6 [75]. The figure was prepared using PyMOL v0.99 [76]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

A primary role of albumin is the binding and transport of long chain fatty acids, that typically circulate in plasma at a total concentration just below 1 mM, 99.9% bound to albumin. Thus, under physiological conditions, ~1–2 fatty acids are carried per albumin molecule. This cargo increases upon exercise and adrenergic stimulation. Several binding sites for fatty acids have been characterized, with the strongest displaying affinity constants of ~10^8 M^{-1} (Fig. 1). The binding of fatty acids brings about a conformational change in the albumin molecule that determines changes in the physicochemical properties, such as an increase in the stability towards heat and proteases [1].

In addition to fatty acids, albumin can bind non-covalently bilirubin, hormones, vitamins, hemin, bile acids and cations, as well as a long list of exogenous compounds. Thus, albumin impacts on the pharmacokinetics of several drugs. The exogenous compounds bind predominantly at two multi-specific high-affinity sites called Sudlow Site I and Site II, located in subdomains IIA and IIIA, respectively (Fig. 1, for recent reviews see [8,9]). This amazing capacity of albumin to bind exogenous compounds is exploited in chromatographic purification protocols involving Cibacron Blue resins, as well as in the traditional clinical laboratory quantification of albumin from biological samples using dyes that shift their absorption properties upon HSA binding.

Some ligands are bound covalently. The single thiol in albumin forms disulfides (HSA-SSR) with the drugs disulfiram or captopril. Analogously, disulfides are formed with the endogenous low molecular weight thiols cysteine, cysteinylglycine, homocysteine and glutathione, as described in detail below. Thioethers (HSA-SR) can be formed with electrophiles. Reactive aldehydes such as 4-hydroxynonenal react preferentially with Cys34 [10]. The thiol can also become S-nitrosated, and S-nitrosoalbumin, detected at concentrations of <0.2 μM [11,12] could constitute a reservoir of nitric oxide and participate in transnitrosation reactions. The thiol may also be a target for nitrated fatty acids through Michael addition reactions leading to novel nitroalkene-thiol adducts [13].

Albumin can also bind cations such as copper(II), nickel(II), zinc(II), cadmium(II), calcium(II), magnesium(II), cobalt(II), gold(I), mercury(II) and platinum(II) in up to four sites with different specificities. The latter cations, particularly gold(I) from antiarthritic drugs, bind at Cys34. The decreased binding of cobalt(II) to albumin has been proposed as a novel marker of cardiac ischemia [14]. This so-called albumin cobalt binding (ACB) test has been approved by the FDA, and its clinical utility is under evaluation. Its putative mechanism is also under scrutiny [15,16].

1.3. Pharmaceutical uses

Albumin therapy is considered for hypovolemia or shock, burns, hypoalbuminemia, surgery or trauma, cardiopulmonary bypass, acute respiratory distress syndrome, hemodialysis, and sequestration of protein-rich fluids. Although used successfully for over 50 years, this relatively expensive therapy remains controversial, and the advantages of albumin over less costly alternative fluids continues to be debated and is the object of multiple randomized controlled trials and meta-analyses (for recent reviews see [17,18]). In addition to these indicated uses, albumin is of considerable pharmacological interest as a drug carrier, through different technologies that include drug conjugates and nanoparticles [19].

Albumin suitable for the administration to patients is obtained from the plasma of donors at >95% purity, filtered and heated after addition of stabilizers (N-acetyltryptophan and sodium octanoate). Recombinant HSA has been successfully produced using different expression systems, most significantly the yeast *Pichia pastoris*. This recombinant albumin shows comparable safety and efficacy

as blood derived albumin [20–22]. The availability of recombinant albumin has opened the way to novel pharmaceutical applications, such as a fusion product of albumin and interferon, currently being assessed in phase III clinical trials for the treatment of hepatitis C [19,23].

2. The thiol of albumin

2.1. Heterogeneity of albumin and its thiol redox status

At least 77 mutations have been identified in the albumin gene [24]. In addition, there are 43 registered cases of analbuminemia that leads to low blood pressure, edema, fatigue, hypercholesterolemia and lipodystrophy (http://www.albumin.org/).

Aside from mutations, in plasma obtained from healthy donors, albumin is heterogeneous, particularly with respect to the redox status of the Cys34 thiol. The predominant form (~70% of total albumin) is reduced mercaptalbumin (HSA-SH), with a theoretical mass of 66,438 Da. Mass spectrometry studies have detected albumin species that are truncated at the N- and C-termini, as well as glycated albumin [25,26] and a novel dehydroalanine modification of Cys487 [27]. At the level of Cys34, the predominant modification observed is cysteinylation, which leads to a mass increase of 119 Da [25,26,28]. This mixed disulfide with cysteine can account for ~25% of total albumin [25,29], while mixed disulfides with other low molecular weight thiols (cysteinylglycine, homocysteine and glutathione) can be detected as well. In addition, a fraction not reducible with dithiothreitol, where the thiol is oxidized to higher oxidation states such as sulfinic (HSA-SO_2H) and sulfonic (HSA-SO_3H) acids, can also be observed [30]. Indeed, through mass spectrometry, a modification with a mass increase of approximately +32 Da has been observed in fresh plasma samples, which can be attributed to oxidation by two oxygens [25,28].

Heterogeneity is more pronounced in commercially available preparations of albumin intended for clinical use [25,28]. The differences between albumin manufacturer and lot number variability may add to the controversy surrounding the administration of albumin and contribute to the conflicting evidence from clinical trials.

2.2. Chromatographic analysis of albumin redox variants

This heterogeneity of albumin can be evidenced through chromatographic procedures. As illustrated in Fig. 2, HPLC using an anion-exchange–hydrophobic interaction column according to modified published procedures [31,32] allowed to separate fresh plasma albumin in three fractions, HSA-1 to HSA-3. These fractions correspond to HSA-SH or mercaptalbumin (HSA-1), HSA-SSR or mixed disulfides reducible with dithiothreitol (HSA-2) and a non-reducible fraction, most likely sulfinic and sulfonic acid (HSA-3). This separation is probably mediated by conformational alterations related to the thiol redox status [31]. Addition of hydrogen peroxide (2 mM, 20 min, room temperature) led to a decrease in HSA-1, which was paralleled by an increase in HSA-3 (13%), consistent with thiol oxidation.

The variations in commercial preparations of purified albumin intended for clinical use are exemplified in Table 1. According to anion-exchange–hydrophobic interaction HPLC, the distribution of albumin redox isoforms differed substantially in samples from two different laboratories (Labs 1 and 2). In fact, the reduced form of albumin, HSA-1, accounted for only 9% of total albumin in the preparation from Lab 2. Treatment with dithiothreitol led to an increase in HSA-1 and a decrease in the HSA-2 fraction (HSA-SSR) as expected, in albumins from both Lab 1 and Lab 2. However, an important fraction was not present as HSA-SSR in the albumin preparations but instead as non-dithiothreitol reducible isoforms within the HSA-3 fraction.

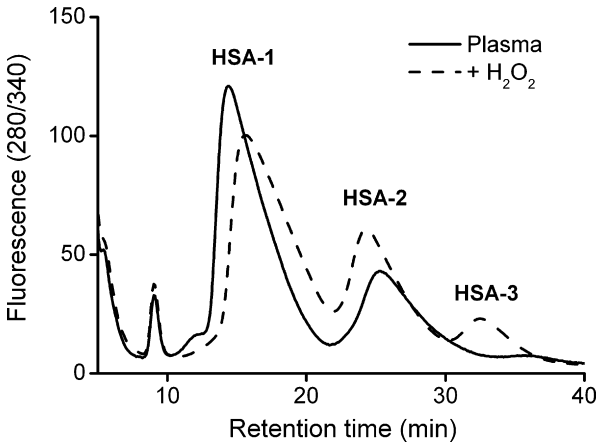

Fig. 2. Anion-exchange–hydrophobic interaction chromatography of plasma. Plasma from a healthy donor was submitted to HPLC using a Shodex Asahipak ES-502N column with an ethanol gradient on 0.4 M sodium sulfate/0.05 M acetate buffer, pH 4.85, according to modified published procedures [31,32]. Fractions HSA-1 (HSA-SH), HSA-2 (HSA-SSR) and HSA-3 (non-dithiothreitol reducible, most likely HSA-SO_2H and HSA-SO_3H) can be identified before (solid line) and after (dashed line) the addition of hydrogen peroxide (2 mM, 20 min).

2.3. Albumin thiol alterations in pathological situations

The advent of potent mass spectrometric techniques applied to albumin, together with the chromatographic analytical approaches described in the previous section, have given rise to a collection of data about redox modifications in different disease conditions, where decreases in reduced mercaptalbumin correlate with increases in oxidized isoforms (mixed disulfides and higher oxidation states). These oxidized species are not present when albumin is secreted from liver cells, thus they may constitute potential markers of the involvement of oxidative stress processes and of the scavenging activity of the albumin thiol. Table 2 compiles data from the literature regarding the detection of oxidized albumin in different conditions and pathological situations.

2.4. Reactivity of the albumin thiol

The thiol of Cys34 can be oxidized by the two-electron oxidants hydrogen peroxide and peroxynitrite. The rate constants of these reactions are 2.2–2.7 and 3.8×10^3 M^{-1} s^{-1} (37 °C, pH 7.4), respectively [33–35]. These values are comparable to the rate constants for the reactions of free cysteine and glutathione, which are 2.9 and 0.87 M^{-1} s^{-1} (37 °C) with hydrogen peroxide and 5.9×10^3 and 1.35×10^3 M^{-1} s^{-1} (37 °C) with peroxynitrite, respectively [36–38].

The albumin thiol is also a target for one-electron oxidants such as hydroxyl radical, carbonate anion radical and nitrogen dioxide. Albumin thiyl radical can be formed as the primary oxidation product and transfer the damage to other residues, forming secondary

Table 1
Redox isoforms of albumin in two preparations intended for clinical use.

Sample	HSA-1 (%)	HSA-2 (%)	HSA-3 (%)
Lab 1	30	50	20
Lab 2	9	48	43
Lab 1 + DTT	75	5	20
Lab 2 + DTT	43	11	46

Commercial preparations of purified albumin intended for clinical use obtained from two different laboratories (Lab 1 and Lab 2) were analyzed. Samples were submitted to anion-exchange–hydrophobic interaction chromatography as described in Fig. 2. HSA fractions 1, 2 and 3 corresponded to reduced thiol (HSA-SH), mixed disulfides (HSA-SSR) and a fraction not reducible with dithiothreitol (DTT), most likely sulfinic and sulfonic acids, respectively. Mean values of duplicate experiments are shown and standard errors are within 5%.

Table 2
Oxidized HSA in pathological conditions and disease states.

Condition	Method	Observation/modification	References
Aging	Chromatography[a]	Decrease in the HSA-SH fraction	[61]
	Thiol measurement	Decrease in the SH/HSA ratio	[62]
	Thiol measurement with HPLC	Decrease in reduced protein thiols	[63]
Focal segmental glomerulosclerosis	Mass spectrometry (LC–ESI-MS/MS for peptides, ESI-MS for whole protein)[b]	Detection of sulfonic acid in HSA	[64,65]
Renal dysfunction	Chromatography[c]	Increase in the oxidized fractions with a decrease of renal function	[66]
Diabetes mellitus	Chromatography[c]	Increase in the oxidized fractions	[67]
Disorders of the temporomandibular joint	Chromatography[c]	In synovial fluid, increase in the oxidized fractions	[32]
Senile cataract	Chromatography[c]	In albumin from the aqueous humor, increase in the oxidized fractions	[68]
Invasive surgery and anesthesia	Chromatography[a]	Increase in the oxidized fractions	[69]
Liver disease (liver cirrhosis, acute-on chronic liver failure)	Chromatography[c]	Increase in the oxidized fractions	[70]
Strenuous exercise	Chromatography[c]	Increase in the oxidized fractions	[71]
Intrauterine growth restriction	Mass spectrometry (LC-ESI-TOF of whole protein)[d]	High levels of cysteinylated maternal HSA	[72]

[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-flight.

radicals. There is also evidence that the thiol can act as a radical sink for other protein radicals, so that its blockage prior to the addition of oxidants leads to increased oxidation of other residues in the albumin molecule [39,40].

Not surprisingly, the reactivity of the albumin thiol is more complex than that of low molecular weight thiols. NMR experiments have suggested that the Cys34 thiol exists in two conformations, and that the reduced thiol predominates in a buried state that shifts to an exposed one upon chemical modification [41,42]. Furthermore, albumin presents pH-dependent structural transitions that can affect the reactivity of the thiol. Thus, the reaction of reduced HSA with the disulfide 2,2′-dithiodipyridine showed, in addition to an increased rate above pH ∼ 7 indicative of thiol deprotonation, an unexpected increase at pH < 5, probably due to a conformational change of the protein [43]. In this context, the pK_a of the albumin thiol remains controversial. Moreover, the binding of fatty acids and the oxidizability of the Cys34 thiol are intimately coupled [44]. Fatty acids lead to an increase in the rate of reaction between the thiol and the disulfide 5,5′-dithiobis(2-nitrobenzoic acid), probably due to changes in the accessibility of the thiol [45]. Indeed, when the three dimensional structures of fatty acid free and lipidated albumin are compared, the increased exposure of Cys34 can be observed (Fig. 3). By using the program AreaIMol from the CCP4 package (Collaborative Computational Project, Number 4), which calculates the solvent accessible surface area, we can estimate that, in the presence of hexadecanoic acid, the water-exposed surface of the sulfur atom of Cys34 is 6.4 Å^2, while 0.7 Å^2 in its absence.

2.5. The albumin thiol as a scavenger of plasma oxidants

Although the albumin thiol does not react particularly fast with oxidants, it can still be considered an important intravascular scavenger due to its very high concentration (0.4–0.5 mM), particularly considering that plasma is scarce in antioxidant defenses and low molecular weight thiols. In this regard, the increase of oxidized isoforms in pathological conditions can be considered as a proof of concept of the scavenger activity of albumin.

Besides albumin, another possible plasma antioxidant could be glutathione peroxidase, but the low concentration of glutathione in plasma (see Table 3) argues against its significance. Importantly, the erythrocyte, loaded with antioxidant enzymes, should be considered an important oxidant scrubber [46–48]. In agreement with the scavenger roles of albumin and of the erythrocyte, Fig. 4 compares the redox status of albumin in oxidation experiments with hydrogen peroxide or peroxynitrite, using either plasma or whole blood. The oxidation of HSA-SH by hydrogen peroxide was highly prevented (∼75%) by erythrocytes, most likely because of the diffusion of hydrogen peroxide into the erythrocyte and its scavenging by the cell's peroxidase/catalase systems, particularly peroxiredoxin [48]. In contrast, when peroxynitrite was the oxidant, the protection exerted by the erythrocytes was significantly lower (∼27%) since, in the presence of physiological concentrations of carbon dioxide and at neutral pH, peroxynitrite is very short lived and forms carbonate anion radical ($CO_3^{\bullet -}$), a more reactive and less diffusible species, along with nitrogen dioxide ($^{\bullet}NO_2$), so that albumin remained the preferential target.

Table 3
Comparison of plasma concentrations of thiols in the reduced or disulfide state with kinetic considerations of albumin sulfenic acid reactivity with reduced thiols.

RSH	[RSH] (μM)[a]	k (M^{-1} s^{-1})[b]	k × [RSH] (×10^{-6} s^{-1})	[Protein mixed disulfides] (μM)[a]	[LMW disulfides] (μM)[a]
Cysteine	9.5	21.6	205	160	104
Cysteinylglycine	2.8	55	151	16	12
Glutathione	4.2	2.9	12	1.3	2.3
Homocysteine	0.24	9.3	2.2	8.8	2.2

[a] Reduced thiol and disulfide concentrations in plasma were taken from [63,73,74]; LMW, low molecular weight.
[b] 25 °C, pH 7.4, reported in [35].

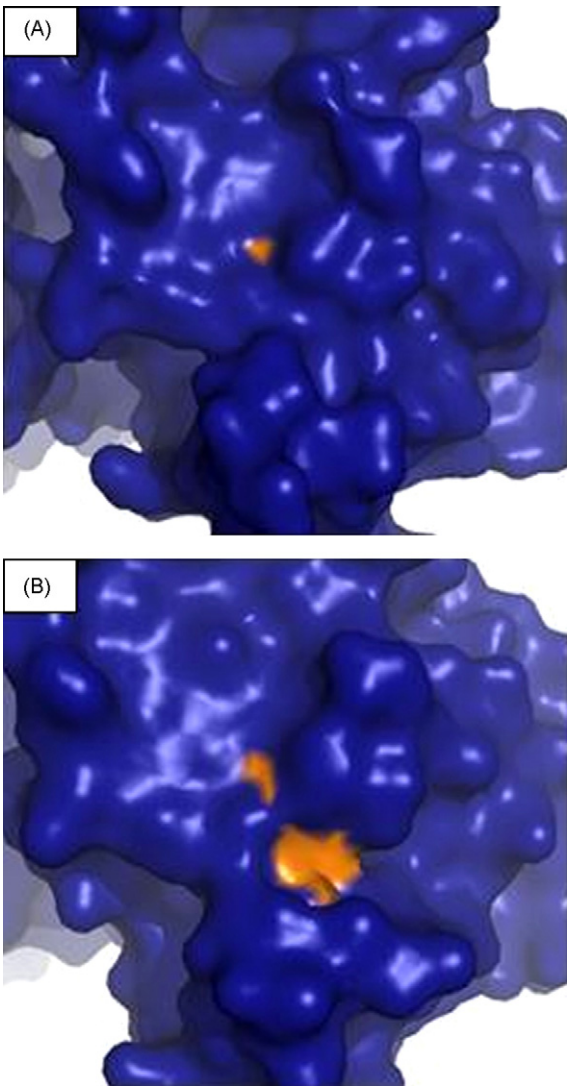

Fig. 3. Surface exposure of the Cys34 site in the absence and in the presence of fatty acids. (A) Lipid-free HSA, Protein Data Bank accession code 1AO6 [75] and (B) HSA with bound hexadecanoic acid, accession code 1E7H [77]. The Cys34 exposed surface is shown in orange. The figures were generated using PyMOL v0.99 [76].

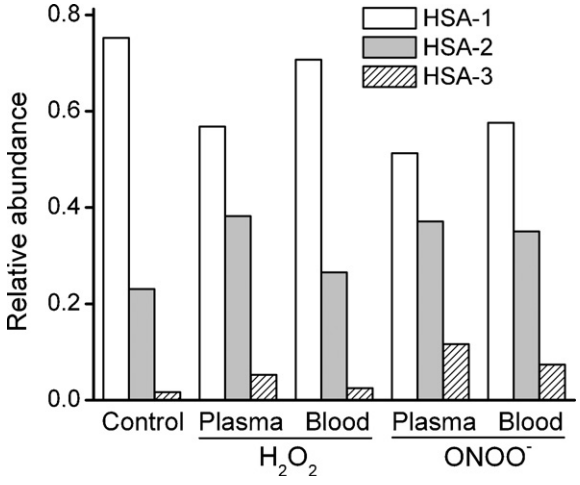

Fig. 4. Modulation of hydrogen peroxide and peroxynitrite-dependent HSA oxidation by erythrocytes. Heparinized human plasma or whole blood was exposed to peroxynitrite (1.5 mM) or H_2O_2 (1.5 mM) for 1.5 h, and oxidative modifications to HSA were analyzed by anion-exchange–hydrophobic interaction chromatography. Mean values of duplicate experiments are shown.

3. Sulfenic acid in human serum albumin

3.1. Detection

The first hint that albumin oxidation led to a product distinct from disulfide came from observations in the 1950s that 1 mol of iodine was needed for the oxidation of 1 mol of albumin thiol, while 0.5 mol would be required for oxidation to a disulfide [49]. The one to one stoichiometry of thiol oxidation was later confirmed for hydrogen peroxide as an oxidant [34]. Accordingly, exposure of albumin to hydrogen peroxide or peroxynitrite did not lead to the formation of intermolecular albumin disulfides (HSA-SS-HSA), probably because of the steric restrictions imposed by the location of Cys34 in a crevice. Evidence for sulfenic acid formation in albumin (HSA-SOH) was obtained from its reaction with sodium arsenite, since this reagent reduces sulfenic acid back to thiol but does not reduce disulfides [36,49,50]. Evidence was obtained too from its reaction with glutathione, since thiols react with sulfenic acid yielding disulfides [34,51]. Although the electrophilic reagent 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) has been used in attempts to detect sulfenic acid in albumin, other nucleophilic groups can also react with it, so NBD-Cl should be used with caution [35,52]. Finally, mass spectrometry of albumin samples oxidized with hydrogen peroxide and treated with the nucleophilic reagent 5,5-dimethyl-1,3-cyclohexanedione (dimedone), a reagent that forms thioethers with sulfenic acid, provided the definitive evidence of sulfenic acid formation at the level of Cys34 [34]. In the control sample of HSA-SH, the tryptic fragment containing Cys34 (residues 21-41, ALVLIAFAQYLQQCPFEDHVK, m/z 2433.9) was detected as the carboxymethylated derivative (m/z 2491.9), since samples were reduced and then submitted to alkylation with iodoacetic acid so as to prevent reactions between the different cysteine containing peptides. MS/MS spectra of the triply charged peptide (m/z 831.4) confirmed the identity of the peptide (Fig. 5A). In the hydrogen peroxide-treated sample, a peptide with a mass increase of 138 Da (m/z 2572.0) could be detected, consistent with the reaction of dimedone with sulfenic acid. MS/MS spectra of the triply charged ion (m/z 857.4) confirmed the identity of the peptide and the position of the dimedone modification at Cys34 (Fig. 5B).

The absence of other thiols in the neighborhood of Cys34 is key to the relative stability of HSA-SOH that facilitates its detection, since sulfenic acids are usually considered transient intermediates. They can react with a thiol forming a disulfide, or with a second sulfenic acid yielding a thiosulfinate, on line with their ability to act both as electrophiles and nucleophiles.

3.2. Reactions

Sulfenic acid can be formed in albumin through the nucleophilic attack of the thiolate on the peroxidic oxygen of hydrogen peroxide and peroxynitrite (Fig. 6). One-electron mechanisms can probably participate in albumin sulfenic acid formation as well, most likely through reaction of thiyl radical with oxygen leading to secondary radicals, including peroxyl radical ($RSOO^{\bullet}$) and sulfinyl radical ($RSO^{\bullet}$), that can finally yield sulfenic acid [53,54]. In fact, the formation of HSA-SOH has been proposed after albumin oxidation with carbonate anion and nitrogen dioxide radicals [54]. Analogously, HSA-SOH has been reported to be formed upon exposure to nitric oxide by a complex mechanism, probably implicating one-electron mechanisms [51].

Due to the absence of UV–vis or fluorescence properties inherent to the sulfenic acid functional group and to its high reactivity in most proteins and low molecular weight compounds, which determine the need to use chemical trapping in its detection, quantitative information regarding sulfenic acid has been particularly

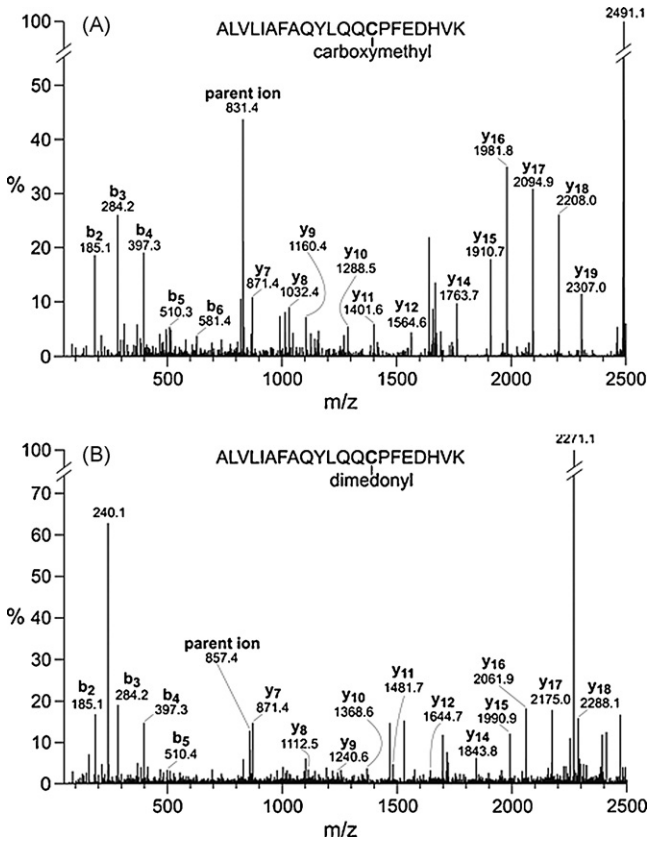

Fig. 5. Mass spectrometric detection of albumin sulfenic acid using dimedone. Control or oxidized (hydrogen peroxide, 1 mM, 30 min) HSA (0.5 mM, 37 °C, pH 7.4) was incubated with dimedone (2.5 mM, 30 min, room temperature) and gel filtered. Samples were reduced with dithiothreitol, alkylated with iodoacetic acid, digested with trypsin and then analyzed through ESI-Q-TOF-MS/MS. (A) Control sample, MS/MS spectrum of the parent fragment *m/z* 831.4 in the triply charged state. (B) Oxidized sample, MS/MS spectrum of the parent fragment *m/z* 857.4 in the triply charged state. Reprinted with permission from [34]. Copyright 2003 American Chemical Society.

elusive to obtain. Our group has successfully approached this problem in the case of albumin using the yellow thiol thionitrobenzoate (TNB) as a probe for sulfenic acid [35]. Indeed, TNB can react with HSA-SOH yielding the mixed disulfide HSA-STNB, with a concomitant decrease in absorbance at 412 nm (Fig. 6). Since the reaction is complex and albumin can also bind non-covalently both TNB and its disulfide DTNB, end point measurements can be misleading. Through the careful kinetic analysis of the reaction, the amount of HSA-SOH formed in albumin after incubation with hydrogen peroxide could be quantified. Using TNB, the rate constants of HSA-SOH reactions with several compounds of analytical and biological interest could be measured using both direct or competition approaches [35].

According to our analysis, HSA-SOH reacted with the probes sodium arsenite and dimedone with rate constants of 0.036 and 0.027 $M^{-1}\,s^{-1}$ (37 °C, pH 7.4), confirming their utility in the detection of sulfenic acid [35].

Our approach also provided valuable insights into the possible biological targets of HSA-SOH. No reaction was detected with the plasma reductants ascorbate and urate, ruling out a role for them in the reduction of sulfenic acid back to thiol, nor with the α-amino groups of amino acids, nor lysine or arginine. Remarkably, although cyclic sulfenamides have been reported to be formed in some proteins from the reaction of sulfenic acid with the neighboring peptidic nitrogen, mass spectrometry yielded no evidence for their formation in albumin, in accordance with the adjacent residue being a proline [35].

Sulfenic acid was able to react with hydrogen peroxide with a rate constant of 0.4 $M^{-1}\,s^{-1}$ (37 °C, pH 7.4). Mass spectrometric analysis of tryptic fragments allowed the identification of a +32 species in Cys34, confirming sulfinic acid (HSA-SO_2H) as product (Fig. 7) [35].

Through competition experiments with TNB, we were able to determine the rate constants for the reactions of HSA-SOH with the thiols present in plasma. These reactions lead to mixed disulfides (Fig. 6). At pH 7.4, HSA-SOH reacted with cysteine, glutathione, homocysteine and cysteinylglycine at 21.6, 2.9, 9.3 and 55 $M^{-1}\,s^{-1}$ (25 °C), respectively [35]. These values provide mechanistic hints into the reaction. First, since the pK_a of the cysteine, glutathione, homocysteine and cysteinylglycine thiols are 8.3, 8.6, 9.9 and 6.9, respectively, it can be concluded that the thiolates are the reactive species towards HSA-SOH, so that the rate constants reflect the proportion of ionized thiol. Second, as expected for a mechanism involving the nucleophilic attack of the thiolate on the sulfenic acid, the intrinsic reactivity of the thiolate (the pH-independent rate constant) increased with pK_a, implying that for these reactions nucleophilicity correlates with basicity (Fig. 8). Remarkably, glutathione deviated from this tendency and reacted particularly slowly with HSA-SOH, probably due to steric restrictions and to its additional anionic charge. Since it has also been reported that glutathione disulfide reacts slowly with HSA-SH and that glu-

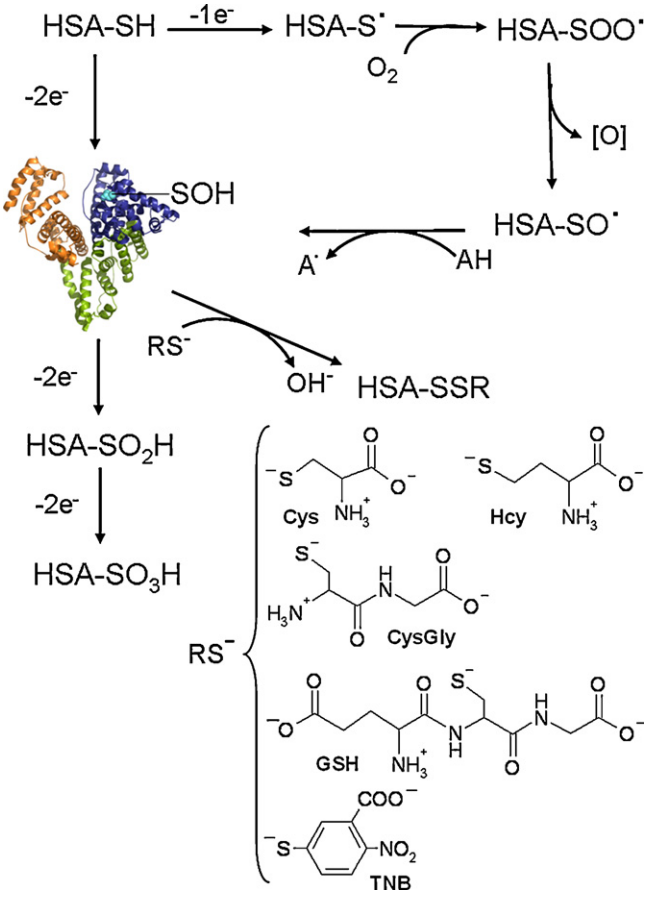

Fig. 6. Formation and reactions of sulfenic acid in human serum albumin. Sulfenic acid (HSA-SOH) can be formed after the two-electron oxidation of the albumin thiol (HSA-SH). One-electron oxidation to thiyl radical (HSA-S•) can lead to secondary radicals, finally yielding HSA-SOH. In the presence of excess oxidant, HSA-SOH can be further oxidized to sulfinic (HSA-SO_2H) and sulfonic (HSA-SO_3H) acid. Alternatively, sulfenic acid can react with another thiolate (RS^-) to form a disulfide (HSA-SSR). The thiols represented are cysteine (Cys, 121.2 Da), homocysteine (Hcy, 135.2 Da), cysteinylglycine (CysGly, 178.2 Da), glutathione (GSH, 307.33 Da) and thionitrobenzoate (TNB, 199.2 Da).

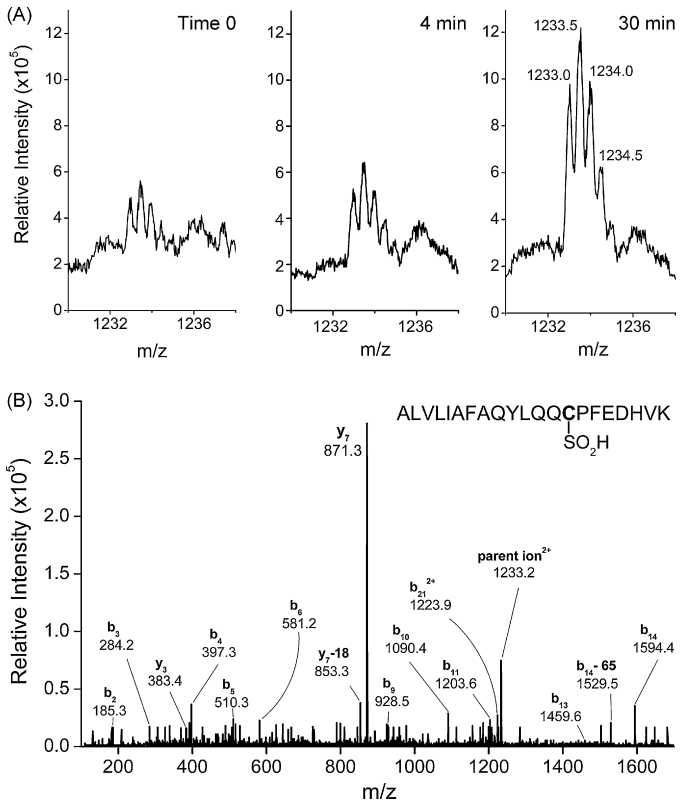

Fig. 7. Mass spectrometric detection of albumin sulfinic acid. (A) HSA (0.5 mM) was incubated with hydrogen peroxide (4 mM, 37 °C, pH 7.4). Reactions were stopped with catalase at 0, 4 and 30 min, samples were digested with trypsin and analyzed by ESI-MS. (B) MS/MS spectrum of the doubly charged parent fragment (m/z 1233.2) corresponding to the Cys34 fragment +32 Da. Reprinted with permission from [35]. Copyright 2008 American Chemical Society.

tathione reacts sluggishly with HSA mixed disulfides [42,55,56], it can be speculated that HSA seems adapted to react slowly with the tripeptide, helping to maintain the thiol/disulfide ratio for plasma glutathione.

3.3. *Albumin sulfenic acid as an intermediate in the formation of mixed disulfides*

With the exception of glutathione, whose thiol/disulfide ratio is ~1–2, the low molecular weight thiols circulate mostly as disulfides, both as low molecular weight disulfides and protein mixed disulfides. The latter are formed mainly (70–90%) with albumin [57]. Several non-enzymatic mechanisms have been proposed for disulfide formation, including radical reactions, thiol-disulfide exchange reactions and intermediacy of sulfenic acid.

A mechanism involving sulfenic acid has been proposed before for albumin, since hydrogen peroxide-treated HSA samples in phosphate buffer (pH 7.4, 37 °C) promoted glutathione oxidation in a process that was inhibited by dimedone and led to albumin–glutathione mixed disulfides and glutathione disulfide [34]. The reactions are:

$$\text{HSA-SOH} + \text{RS}^- \rightarrow \text{HSA-SSR} + \text{OH}^- \quad (1)$$

$$\text{HSA-SSR} + \text{RS}^- \rightarrow \text{HSA-S}^- + \text{RSSR} \quad (2)$$

This mechanism for disulfide formation is compatible with the kinetic data obtained by our group regarding the reactivity of the different plasma thiols with HSA-SOH leading to mixed disulfides. Table 3 shows the extrapolation of our kinetic data to the plasma situation. A rough correlation can be appreciated between the kinetic factors obtained by multiplying the second-order rate constant and the thiol concentration for each low molecular weight thiol (i.e. the apparent first-order rate constants) with the abundance of plasma mixed disulfides, consistent with a role for albumin sulfenic acid in disulfide formation. In this regard, the most abundant mixed disulfide corresponds to cysteine, whose thiol is in turn the fastest reacting under plasma conditions. Remarkably, the slowest reaction is estimated for homocysteine. However, the disulfides of homocysteine circulate at relatively high concentrations, probably reflecting the tendency of homocysteine to form disulfides due to its high pK_a and the contribution of additional mechanisms such as thiol-disulfide exchange.

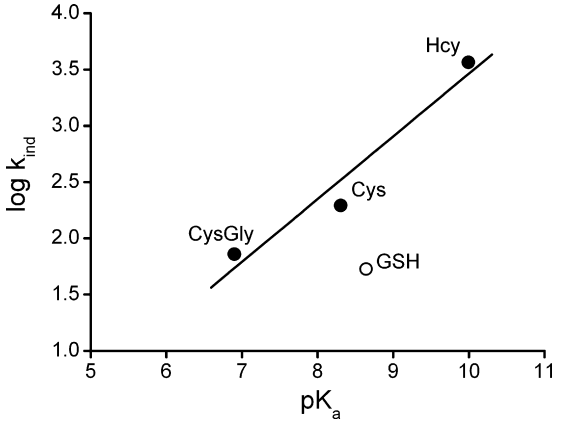

Fig. 8. Brønsted-type plot for the reaction of the thiolates present in plasma with HSA-SOH. Rate constants at pH 7.4 and 25 °C with cysteinylglycine (CysGly), cysteine (Cys), glutathione (GSH) and homocysteine (Hcy) were determined using a competition approach with TNB in [35]. The pH-independent rate constants (k_{ind}) were calculated from the rate constants at pH 7.4 ($k_{7.4}$) according to the equation $k_{7.4} = k_{ind}\ K_a/(K_a + [H^+])$ using reported pK_a values. The slope of the plot yielded a Brønsted coefficient of $\beta = 0.56$.

The formation of albumin mixed disulfides can be potentially reverted with suitable reductants. In this sense, the reaction of HSA-SSR with a low molecular weight thiol (Eq. (2)) can occur and leads to a low molecular weight disulfide, regenerating HSA-SH [34,58].

4. Concluding remarks—albumin sulfenic acid is a likely player in the determination of the plasma redox status

It is our proposal that albumin sulfenic acid is a key intermediate in plasma oxidation processes, linking oxidant species with final oxidation products, so that its reactions participate in the determination of the plasma redox status. According to our kinetic studies regarding the reactivity of albumin sulfenic acid, the preferred kinetic fate of HSA-SOH formed in plasma would be the reaction with low molecular weight thiols to form mixed disulfides. These are observed in the circulation and actually increase under certain disease states. Alternatively, HSA-SOH can be further oxidized to sulfinic and sulfonic acids, a so far irreversible modification that is also augmented in different pathologies (Table 2).

As exemplified from the numbers in Table 3, the different thiol and oxidized species are not in equilibrium [59,60], implying that kinetic barriers exist for maintaining their concentrations. The mechanisms involved are only starting to be unraveled, and albumin sulfenic acid is a likely player in the determination of the intravascular redox status.

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