Amino Acids (2007) 32: 543–551
DOI 10.1007/s00726-006-0430-y
Printed in The Netherlands

Amino Acids

Sulfenic acid in human serum albumin

Review Article

S. Carballal[1,2,3], **B. Alvarez**[1,3], **L. Turell**[1,3], **H. Botti**[2,3], **B. A. Freeman**[4], and **R. Radi**[2,3]

[1] Laboratorio de Enzimología, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay
[2] Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay
[3] Center for Free Radical and Biomedical Research, Universidad de la República, Montevideo, Uruguay
[4] Department of Pharmacology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA

Received June 10, 2006
Accepted June 30, 2006
Published online October 24, 2006;

Summary. Sulfenic acid (RSOH) is a central intermediate in both the reversible and irreversible redox modulation by reactive species of an increasing number of proteins involved in signal transduction and enzymatic pathways. In this paper we focus on human serum albumin (HSA), the most abundant plasma protein, proposed to serve antioxidant functions in the vascular compartment. Sulfenic acid in HSA has been previously detected using different methods after oxidation of its single free thiol Cys34 through one- or two-electron mechanisms. Since recent evidence suggests that sulfenic acid in HSA is stabilized within the protein environment, this derivative represents an appropriate model to examine protein sulfenic acid biochemistry, structure and reactivity. Sulfenic acid in HSA could be involved in mixed disufide formation, supporting a role of HSA-Cys34 as an important redox regulator in extracellular compartments.

Keywords: Thiol – Human serum albumin – Sulfenic acid – Peroxynitrite – Hydrogen peroxide – Free radicals

Abbreviations: HSA, human serum albumin; HSA-SH, the thiol of HSA; BSA, bovine serum albumin; SH/HSA, amount of thiol per albumin molecule; RSOH, sulfenic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); dimedone, 5,5-dimethyl-1,3-cyclohexanedione; GSH, glutathione; GSSG, glutathione disulfide.

Introduction

The overproduction of reactive oxygen species including nitrogen oxides under conditions of oxidative stress may damage various biomolecules leading to cell and tissue toxicity. In addition, there is increasing evidence that these reactive mediators play a central role in signal transduction under physiological and physiopathological conditions, leading to new proposals on the definition of oxidative stress in terms of disruption of redox signaling and control (Hansen et al., 2006a).

Thiols are preferential targets of reactive species. With one- and two-electron reduction potentials $E^{\circ\prime}$ ($RS^{\bullet}/RSH$) of 0.92 V and $E^{\circ\prime}$ (RSSR/2RSH) of −0.24 V for cysteine (Surdhar and Armstrong, 1986; Keire et al., 1992), thiols can be oxidized by a wide spectrum of radical and non-radical species, usually at rates several orders of magnitude faster than other amino acids. At the high concentrations that thiols can achieve in different cell compartments, thiols can effectively compete with other targets. Last, several thiol oxidation products can be efficiently repaired.

In the last few years, the reactions of reactive species with thiols, far from constituting simple scavenging pathways, are being recognized to participate in modulatory and signaling mechanisms, coupling changes in the redox state of the milieu to biochemical and cellular processes (Schafer and Buettner, 2001). Among the different posttranslational modifications generated by thiol oxidation, sulfenic acid is receiving increased interest since it has been identified in a growing list of proteins, where it serves catalytic or regulatory functions. In this paper we will focus on the formation of sulfenic acid in human serum albumin and revise its properties and possible biological function.

Thiol oxidation states and sulfenic acid formation

Oxidation of low molecular weight or protein thiols typically results in the formation of disulfide bonds (RSSR).

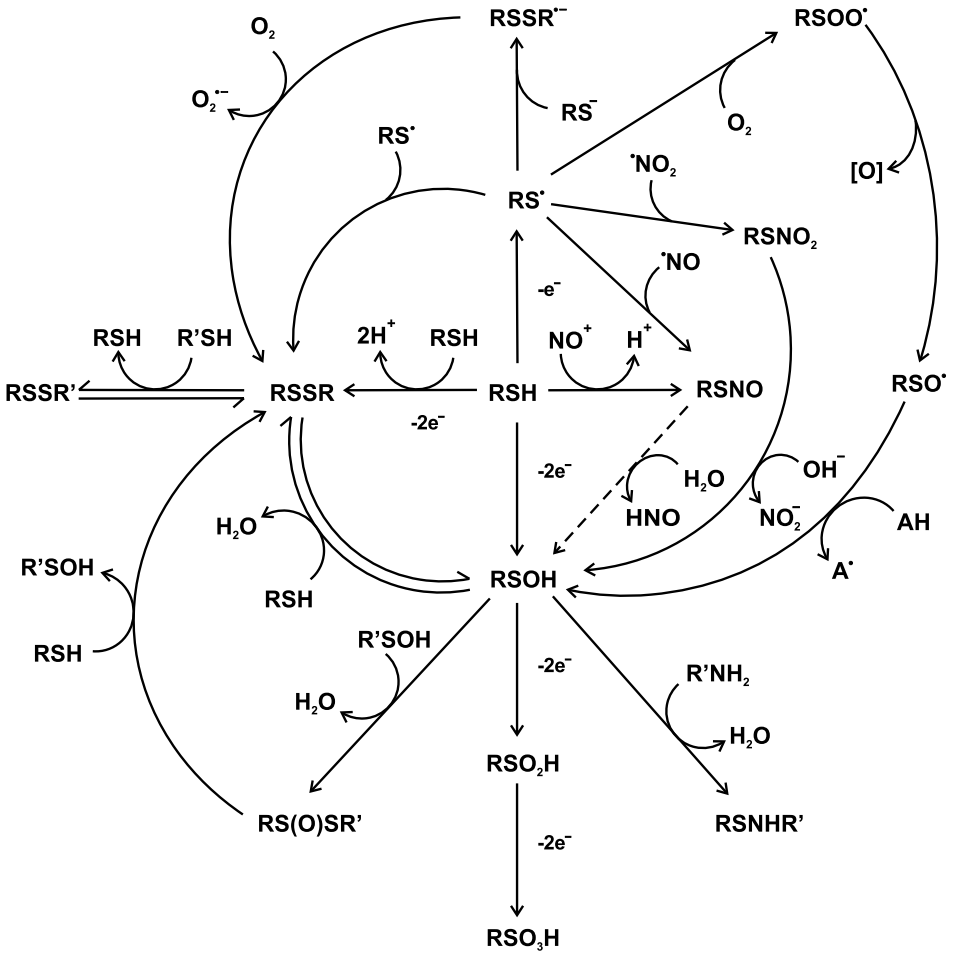

Fig. 1. Pathways of thiol oxidation by reactive species. Thiols can be oxidized in a two-electron oxidation pathway beyond disulfide (RSSR) to yield sulfenic acid (RSOH) or, in the presence of excess oxidant, can be further oxidized to sulfinic (RSO_2H) or sulfonic (RSO_3H) acid derivatives. Disulfide can undergo a thiol-disulfide exchange reaction with another thiol group to yield a mixed disulfide (RSSR') and a reduced thiol. Sulfenic acid can react with another thiol to form a disulfide, which can be reduced by suitable reductants as in the catalytic cycle of peroxiredoxins. Alternatively, it can react with a second sulfenic acid to yield thiosulfinate (RS(O)SR), which is able to react with another thiol to form a disulfide and regenerate the sulfenic acid. In addition, sulfenic acid can react with amines to form sulfenamide (RSNHR'). One-electron oxidation pathways can yield thiyl radical ($RS^{\bullet}$) which can dimerize with another thiyl radical to form disulfide or react with a thiolate yielding the disulfide radical anion ($RSSR^{\bullet-}$) that in the presence of oxygen can promote the formation of disulfide and superoxide radical anion ($O_2^{\bullet-}$). Alternatively, thiyl radical can initiate an oxygen-dependent chain reaction to produce a number of secondary radicals, including peroxyl radical ($RSOO^{\bullet}$) and sulfinyl radical ($RSO^{\bullet}$) that can finally yield sulfenic acid. Thiols can also become oxidized by reactive nitrogen species to yield nitrosothiol (RSNO) and nitrothiols ($RSNO_2$) that may also lead to sulfenic acid formation. For simplicity, general formulas are presented instead of pH-dependent variants (*i.e.* RSH instead of RS^-)

Extracellular proteins often rely on disulfide bonds to support their correct folding and maintain their structural stability. In contrast, intracellular proteins usually contain reduced cysteine due to the highly reducing environment.

Sulfenic acids (RSOH) are key intermediates in thiol oxidation processes (Fig. 1). The best understood pathway for sulfenic acid formation is the reaction of a thiol with different oxidants such as hydrogen peroxide and peroxynitrite where the thiol changes its formal oxidation state by +2. Sulfenic acids are usually unstable and can react with a reduced thiol yielding a disulfide. For example, the reaction of a protein sulfenic acid with glutathione (GSH), the principal cytosolic low molecular weight thiol, is one of the mechanisms that can lead to the formation of glutathionylated proteins (RSSG). Alternatively, sulfenic acid can react with a second sulfenic acid to yield thiosulfinates (RS(O)SR); or in the presence of excess oxidant, it can be further oxidized to sulfinic (RSO_2H) or sulfonic (RSO_3H) acids (Torchinsky, 1981; Giles and Jacob, 2002; Paget and Buttner, 2003). This picture gains further levels of complexity since sulfenic acid can also react with amines to form sulfenamides (RSNHR) (Allison, 1976; Raftery et al., 2001). Indeed, recent studies identified a cyclic sulfenamide species formed on the sulfenic acid of protein tyro-

sine phosphatase 1B after reaction with an amide nitrogen of a neighboring residue (van Montfort et al., 2003; Salmeen et al., 2003; Salmeen and Barford, 2005).

Alternatively, thiol oxidation by one-electron pathways results in a sulfur-centered radical (thiyl radical, $RS^{\bullet}$) which is highly reactive and can combine with another thiyl radical to form disulfides, or react with a thiolate to yield a reductive disulfide radical anion ($RSSR^{\bullet-}$) that forms superoxide upon reaction with molecular oxygen. Thiyl radicals have also been shown to react with oxygen to form the peroxyl radical ($RSOO^{\bullet}$), an unstable intermediate that rapidly derives in sulfinyl radical ($RSO^{\bullet}$) which after hydrogen abstraction from reductants (AH) can finally yield sulfenic acid through a free radical pathway (Quijano et al., 1997; Bonini and Augusto, 2001; Giles and Jacob, 2002).

In addition to the oxyacids listed above, thiol exposure to reactive species derived from nitric oxide can also yield nitroso (RSNO) and presumably nitrothiols ($RSNO_2$), which can also be sulfenic acid precursors (Stamler et al., 1992; Wink et al., 1994; van der Vliet et al., 1998).

Undoubtedly, several of the products of thiol oxidation by reactive species are highly reactive themselves (*i.e.* $RS^{\bullet}$, RSOH). This has led to the definition of an additional group of redox active molecules named reactive sulfur species (RSS), complementary to the list of reactive oxygen and nitrogen species (Giles and Jacob, 2002).

The formation of disulfides, sulfenic acid and nitrosothiol can be reverted to thiol non-enzymatically or enzymatically, with the aid of the glutaredoxin and thioredoxin systems, or to glutathione disulfide by GSNO reductase (Liu et al., 2001; Sahoo et al., 2006). In contrast, the formation of higher oxidation states such as sulfinic (RSO_2H) and sulfonic acids (RSO_3H) has been considered to be irreversible, although recent evidence indicates that reduction of the sulfinic form of several peroxiredoxins can occur enzymatically *in vivo* by cysteine sulfinyl reductases in a rather complex ATP-consuming mechanism (Biteau et al., 2003; Budanov et al., 2004).

Sulfenic acid stabilization and reactivity

Sulfenic acids exhibit both nucleophilic and electrophilic reactivity in biological milieus. Consequently, sulfenic acid can react with another thiol providing a mechanism for disulfide formation or condense with a second sulfenic acid to form a thiosulfinate (RS(O)SR) (Kice and Cleveland, 1973; Torchinsky, 1981; Claiborne et al., 1993). Since there are few electrophilic functional groups in proteins, the fact that nucleophiles can react with the sulfur atom of sulfenic acid is remarkable.

The biological formation of low molecular weight sulfenic acid is difficult to detect due to its high reactivity and consequent instability, therefore it has long been thought to be a transient intermediate in the oxidation of thiols. More recently, however, the formation of a relatively stable sulfenic acid has been identified in several proteins and enzymes and is receiving increased interest since it has been recognized to play a crucial role in catalytic, redox regulatory and signaling functions (Claiborne et al., 1999, 2001; Finkel, 2000). Indeed, sulfenic acid is involved in the catalytic cycle of several enzymes. Peroxiredoxins, involved in peroxide detoxification, contain a conserved cysteine that in the catalytic cycle is oxidized to a sulfenic intermediate, which then condenses with another cysteine to form a thioredoxin-reducible disulfide. In addition to this catalytic cycle, sulfenic acid can also be overoxidized to sulfinic acid in an inactivation process that can be eventually reverted by cysteine sulfinyl reductases.

Sulfenic acid stabilization depends on steric hindrance and the local environment of the thiol involved. The most important factor to account for sulfenic acid stabilization is the absence of other proximal thiol groups, as well as intramolecular hydrogen bonding interactions with suitable hydrogen bonding partners, ionization of sulfenic acid to stabilize the corresponding sulfenate, together with an apolar microenvironment with limited solvent access (Claiborne et al., 1999).

Human serum albumin

Human serum albumin (HSA) is the most abundant protein in the vascular compartment (0.6 mM) where it represents ~60% of the total proteins in plasma. It is interesting to note that an important amount of albumin is localized extravascularly (Peters, 1996). Actually, the albumin pool present in extravascular/extracellular compartments represents more than double of the intravascular pool. This globular protein of 66 kDa consists of a non-glycosylated single chain polypeptide containing 585 amino acids cross-linked by 17 disulfide bridges with one tryptophan, six methionines, 18 tyrosines and only one free cysteine, Cys34. The crystallographic structure of HSA with its reduced cysteine has already been resolved (Carter et al., 1989; Carter and He, 1990). Heart-shaped, it contains 67% alpha-helix and no beta-sheet with three homologous domains that contain common structural motifs (Fig. 2).

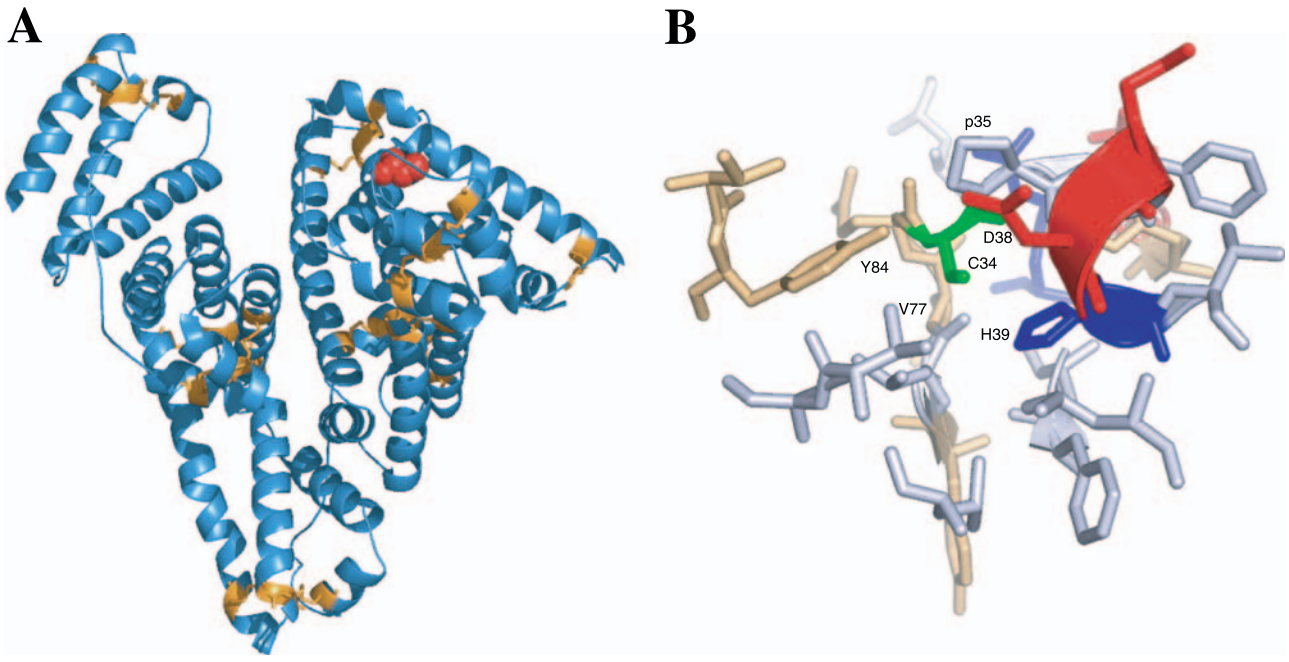

Fig. 2. Three-dimensional structure of HSA and location of Cys34. **A** Domain structure of human serum albumin. The locations of the disulfide bonds are shown in orange and Cys34 is shown in red. The amino terminus is at the right and the carboxyl terminus at the left. **B** Structural features of groups within 8 Å of the Cys34 site showing Asp38, Pro35, His39, Val77 and Tyr84. Polar residues are shown in light orange and non polar residues are shown in light blue. Cys34 is shown in green. Polar hydrophilic residues with positive and negative charge are colored in red and blue, respectively. HSA structural data was downloaded from the Protein Data Bank, accession code 1BMO (Sugio et al., 1999) and the figure was generated using PyMOL Molecular Graphics System (DeLano, 2002)

In addition to its physiological functions in plasma that include the maintenance of colloid osmotic pressure and the binding and transport of several ligands such as fatty acids, hormones and drugs, albumin is also proposed to serve antioxidant functions in the vascular compartment. This protective role is mainly attributed to its single thiol, Cys34, which accounts for ~80% of reduced thiols in human plasma and was determined to be the preferential plasma scavenger of the diverse family of oxygen and nitrogen reactive species (Halliwell, 1988; Halliwell and Gutteridge, 1990; Radi et al., 1991a; Alvarez et al., 1999).

Albumin is heterogeneous with respect to the thiol content. In plasma samples, the main HSA fraction called mercaptalbumin contains the reduced free thiol (~70% of total HSA). An important fraction of non-mercaptalbumin contains mixed disufides between albumin thiol (HSA-SH) and low molecular weight thiols. In fact, about one-fourth of circulating albumin is present as mixed disulfides with cysteine, cysteinylglycine, homocysteine, γ-glutamylcysteine or glutathione. It has been proposed that in the remaining plasma non-mercaptalbumin fraction, the thiol is oxidized to higher oxidation states including sulfinic and sulfonic acids non-reducible by thiol reagents (Janatova et al., 1968; Noel and Hunter, 1972; Era et al., 1988).

This balance between reduced and oxidized forms of HSA can shift in pathological states (Kawai et al., 2001; Tomida et al., 2003), with intense exercise (Inayama et al., 1996; Imai et al., 2002) and during ageing (Leto et al., 1970; Era et al., 1995). For example, an age-dependent decrease in the amount of thiol per human serum albumin molecule (SH/HSA) was observed, consistent with oxidative age-related damage.

Reactivity of HSA-SH with reactive species

Previous work from our group showed that the thiol in albumin was the preferential plasma target of the reactive species generated by xanthine oxidase, which include superoxide, hydrogen peroxide and hydroxyl radical (Radi et al., 1991b). We also demonstrated that among all amino acids, the thiol was the preferential target of peroxynitrite and its derived radicals (Radi et al., 1991a; Alvarez et al., 1999).

The thiol in HSA reacts with hydrogen peroxide and peroxynitrite with rate constants of 2.26 and $3.8 \times 10^3\,M^{-1}\,s^{-1}$ at 37 °C and pH 7.4 (Alvarez et al., 1999;

Carballal et al., 2003). This reactivity is comparable to that of low molecular weight thiols such as cysteine, glutathione and homocysteine (Radi et al., 1991a; Koppenol et al., 1992; Winterbourn and Metodiewa, 1999; Trujillo and Radi, 2002). Unlike other proteins that have thiols several orders of magnitude more reactive, enhanced by the protein microenvironment and their low $pK_{A_{SH}}$ values, such as peroxiredoxins or tyrosine phosphatases (Takakura et al., 1999; Bryk et al., 2000; Trujillo et al., 2004), the thiol in HSA is not particularly reactive exhibiting an apparent $pK_{A_{SH}}$ of 8.3–8.6, similar to those of cysteine (8.36) and glutathione (8.75) (Wilson et al., 1980; Torchinsky, 1981). Although the thiol in HSA is oxidized at relative slow rates, it still constitutes an important scavenger due to its high concentration in plasma (0.4–0.5 mM).

The oxidation of HSA-SH with hydrogen peroxide and peroxynitrite did not lead to the formation of disulfide bridged HSA dimers (Radi et al., 1991b; Carballal et al., 2003). Steric restrictions and the location of the single thiol of albumin in a crevice preclude dimer formation. This suggested that HSA-SH was oxidized to higher oxidation states such as sulfenic acid or further oxidized to sulfinic or sulfonic acids.

Sulfenic acid detection in HSA

Since sulfenic acids do not possess distinguishing absorbance or fluorescence features and are unstable, different tools and chemical methods have been developed for trapping and identifying such functional groups.

An important characteristic of sulfenic acid is the reactivity towards nucleophilic reagents such as benzylamine or dimedone (5,5-dimethyl-1,3-cyclohexanedione), that react specifically with sulfenic acid to form a stable thioether (Allison, 1976). These reagents can only be identified by mass spectrometry or using the radioactively labeled compounds. Recently, modified dimedones have been designed that react with sulfenic acids yielding fluorescent signals (Poole et al., 2005). While this reaction remains the standard for detection of sulfenic acids, reactions of dimedone with other groups such as aldehydes may be possible, therefore mapping the alkylation to a specific cysteine residue is strongly recommended.

Sulfenic acid can also be determined using thionitrobenzoate, another nucleophilic reagent that forms mixed disulfides and results in a loss in the absorbance at 412 nm (Ellis and Poole, 1997b; Boschi-Muller et al., 2000; Peshenko and Shichi, 2001).

The electrophilic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl) has also been employed to detect sulfenic acid in proteins (Ellis and Poole, 1997a; Denu and Tanner, 1998; Carballal et al., 2003), but its ability to react with other protein nucleophile groups is a matter of concern.

Last, sulfenic acid can be detected after its reaction with arsenite, since arsenite reduces sulfenic acid back to thiol but does not reduce disulfides. Recently, proteomic approaches have been devised that allow to detect sulfenic acid based on its specific reduction with arsenite, followed by thiol detection with biotin-maleimide (Saurin et al., 2004).

The formation of sulfenic acid in albumin was first suggested by arsenite reduction of hydrogen peroxide-oxidized BSA-SH (Radi et al., 1991a, b). Later, its formation was also proposed after exposure of HSA to nitric oxide (DeMaster et al., 1995) as well as platinum complexes (Kratochwil et al., 1999). More recently mass spectral analyses using dimedone confirmed that sulfenic acid was formed by hydrogen peroxide-oxidation of HSA Cys34 and provided the definitive evidence for the formation of this derivative in human albumin (Carballal et al., 2003).

The reaction of sulfenic acid with thiols such as glutathione to form disulfides is a useful tool for characterizing the formation of sulfenic acid in HSA. Indeed, in the experiment described in Table 1 we show that, when peroxynitrite-treated HSA was incubated with reduced glutathione, the concentration of the latter decreased in the ultrafiltrates due to its reaction with sulfenic acid, in line with the initial formation of HSA-SOH by peroxynitrite which oxidizes thiols by two electrons. In the presence of carbon dioxide peroxynitrite decomposes to produce nitrogen dioxide and carbonate radical anion ($CO_3^{\bullet -}$) in

Table 1. Oxidation of GSH by peroxynitrite and peroxynitrite/CO_2-treated HSA

Time (min)	GSH oxidized (nmol)[b]	
	$-CO_2$	$+CO_2$
3[a]	156 ± 7[b]	85 ± 8
60	152 ± 20	70 ± 15
120	157 ± 28	56 ± 11

[a] The amount of HSA thiols decreased upon exposure to peroxynitrite in the absence and in the presence of CO_2 from 0.563 mM to 0.187 mM and 0.357 mM, respectively

[b] The results are expressed as mean ± S.D. ($n = 3$)

HSA (0.56 mM) was incubated with $ONOO^-$ (1 mM) in the presence and absence of CO_2 (25 mM $NaHCO_3$) at 37 °C, pH 7.4. At different time points, aliquots (0.5 mL) were removed and incubated with glutathione (0.48 mM) for 30 min. Then, the samples were ultrafiltered and glutathione oxidation was determined as reported previously (Carballal et al., 2003). As control, the addition of decomposed peroxynitrite to HSA led to no thiol oxidation. Catalase was added to the aliquots to prevent oxidation of glutathione by residual hydrogen peroxide

35% yield. These species can oxidize HSA-SH to thiyl radical (RS$^{\bullet}$) which has been detected previously through EPR during the oxidation of BSA and low molecular weight thiols (Gatti et al., 1994; Bonini and Augusto, 2001) and can initiate an oxygen-dependent radical chain reaction. Remarkably, peroxynitrite-treated HSA in the presence of carbon dioxide also led to a decrease in the concentration of reduced glutathione, in agreement with the formation of sulfenic acid under these conditions, although to a lower extent. In Fig. 1 we show that the peroxyl radical derived from the reaction of thiyl radical with oxygen (RSOO$^{\bullet}$) can undergo homolytic cleavage to a sulfinyl radical (RSO$^{\bullet}$) which can then oxidize another residue and finally yield sulfenic acid. Thus, our results support the hypothesis that sulfenic acid in HSA can also be formed through a free radical pathway.

Stability of sulfenic acid in HSA

Although sulfenic acids are generally unstable and highly reactive, HSA-SOH generated from exposure of albumin to peroxynitrite was remarkably stable (Table 1), similar as reported for hydrogen peroxide-treated HSA where more than 85% of sulfenic acid formed remained after 120 min (Carballal et al., 2003). In the presence of carbon dioxide, sulfenic acid was less stable, probably due to the free radical mechanism involved. Since stabilization depends on the local environment of the thiol in the native protein, the three-dimensional structure of HSA provided by X-ray diffraction confirms that Cys34 is located in a partially solvent-protected cleft in a hydrophobic crevice of 9.5–10 Å with no other adjacent thiol, surrounded by side chains of Pro35 and Val77 which could help to limit solvent accessibility and contribute to the apolar microenvironment. In addition, the imidazole ring from His39, the carboxylate group of Asp38 and the hydroxyl group of Tyr84 could also stabilize sulfenic acid with suitable electrostatic and hydrogen bonding interactions (Fig. 2) (He and Carter, 1992; Sugio et al., 1999; Stewart et al., 2005). It is possible that sulfenic acid will be present as the sulfenate anion at neutral pH, since its p*K*A is expected to be lower than that of the thiol, based on the p*K*As of the oxyacids of cysteine (2.1 and 1.3 for cysteine sulfinic and sulfonic acids, respectively) and those reported for stable low molecular weight sulfenic acids (4.8–6.3) (Claiborne et al., 1993).

Sulfenic acid in the context of the plasma environment

In contrast to the intracellular situation, where a reducing environment is maintained by a high concentration of glutathione (5–10 mM) in the reduced state (GSH/GSSG ~100), the extracellular plasma environment is more oxidizing, with total ratios of reduced/oxidized thiols close to 1–2.

The most abundant reduced thiol in plasma is that of HSA (400–500 μM), while the concentration of reduced low molecular weight thiol compounds only totals 10–20 μM. Indeed, low molecular weight thiol compounds such as cysteine (265 μM), cysteinylglycine (32 μM), homocysteine (12 μM) and glutathione (7.5 μM) are present at total concentrations of ~300 μM and mostly oxidized, with ratios of reduced *versus* total disulfides close to 0.01–0.1. The exception is glutathione, where the ratio reduced/total disulfide is close to 2 (5.1 μM GSH versus 1.5 μM low molecular weight glutathione disulfides and 1.6 μM glutathione protein disulfides) (Mansoor et al., 1992; Andersson et al., 1993). In turn, a very important fraction of the low molecular weight thiol compounds (65%) are forming mixed disulfides with proteins, mainly with HSA, so that the reduced/disulfide ratio is close to 3 for HSA.

Thus, it is interesting to note that, analogously to the intracellular situation, the different plasma thiol/disulfide pools are not in equilibrium, and therefore could function as control nodes for different redox sensitive processes (Hansen et al., 2006b), although the kinetic barriers accounting for such differences are not yet elucidated. It is most likely that exchanges with intracellular compartments probably play a critical role in maintaining the thiol pools, since it has been observed that cultured cells and perfused tissues are able to regulate the redox state of extracellular compartments (Moriarty-Craige and Jones, 2004; Hansen et al., 2006a). In this line of thought, it is interesting to note that oxidized conformationally denatured albumin, which occurs *in vitro* and under oxidative stress conditions, is taken up by endothelial cells at increased rates (Schnitzer et al., 1992; Bito et al., 2005).

The numbers given above highlight the fact that the albumin thiol will be an important scavenger of reactive species, while low molecular weight thiols will not be able to compete with albumin for oxidants. This scavenging by Cys34 could represent a catalytic activity if the oxidation of the thiol in HSA is reverted by suitable yet undetermined reductants. In addition, it has been recently reported that the HSA disulfide between Cys392 and Cys438 sustains lipid peroxidase activity *in vitro* in the presence of thioredoxin (Cha and Kim, 2006), but its physiological significance in plasma needs to be confirmed. Another possible target for peroxides will be plas-

matic glutathione peroxidase, but since glutathione is scarce, the system may be easily overcome (Brigelius-Flohe, 1999).

While albumin circulates mostly in the reduced state, it can certainly form mixed disulfides. Assuming that albumin is released into the circulation from the liver with Cys34 mostly in its reduced form (Peters, 1996), the fact that 25% of HSA circulates as mixed disulfides with low molecular weight thiols, mainly cysteine, can be accounted for by three possible non-enzymatic mechanisms: thiol-disulfide exchange, radical reactions or reactions of sulfenic acid with a thiol (Di Simplicio et al., 2005). Indeed, one possible role for the stable sulfenic acid in albumin could be as intermediate in mixed disulfide formation (Carballal et al., 2003).

Sulfenic acid in HSA as well as mixed disulfides, could represent reversible modifications able to regenerate reduced HSA thiol with the appropriate reductant. This can be considered a protective mechanism to prevent overoxidation of cysteine to sulfinic and sulfonic acid. The detection of sulfenic acid *in vivo* remains a challenge. Also, whether an enzymatic system is operative in plasma for reducing sulfenic acid or mixed disulfides, and what the ultimate reductants are, needs to be established. Hopefully, the characterization and quantification of the oxidative modifications in albumin Cys34 will have utility as biomarkers of oxidative reactions in the vascular compartment and will yield useful information concerning the role and/or significance of the stable sulfenic acid in HSA.

Acknowledgements

We thank Drs. Madia Trujillo, Gerardo Ferrer-Sueta, and Martín Graña (Universidad de la República) for helpful comments. This work was supported by grants from Programa de Desarrollo Tecnológico-II (Ministry of Culture and Education, Uruguay) and Comisión Sectorial de Investigación Científica (Universidad de la República, Uruguay) (to B.A.), the Philip Morris External Research Program (to B.A.F.) and the Howard Hughes Medical Institute (to R.R.). R.R is a Howard Hughes International Research Scholar.

References

Allison WS (1976) Formation and reactions of sulfenic acids in proteins. Acc Chem Res 9: 293–299

Alvarez B, Ferrer-Sueta G, Freeman BA, Radi R (1999) Kinetics of peroxynitrite reaction with amino acids and human serum albumin. J Biol Chem 274: 842–848

Andersson A, Isaksson A, Brattstrom L, Hultberg B (1993) Homocysteine and other thiols determined in plasma by HPLC and thiol-specific postcolumn derivatization. Clin Chem 39: 1590–1597

Biteau B, Labarre J, Toledano MB (2003) ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. Nature 425: 980–984

Bito R, Hino S, Baba A, Tanaka M, Watabe H, Kawabata H (2005) Degradation of oxidative stress-induced denatured albumin in rat liver endothelial cells. Am J Physiol Cell Physiol 289: C531–C542

Bonini MG, Augusto O (2001) Carbon dioxide stimulates the production of thiyl, sulfinyl, and disulfide radical anion from thiol oxidation by peroxynitrite. J Biol Chem 276: 9749–9754

Boschi-Muller S, Azza S, Sanglier-Cianferani S, Talfournier F, Van Dorsselear A, Branlant G (2000) A sulfenic acid enzyme intermediate is involved in the catalytic mechanism of peptide methionine sulfoxide reductase from Escherichia coli. J Biol Chem 275: 35908–35913

Brigelius-Flohe R (1999) Tissue-specific functions of individual glutathione peroxidases. Free Radic Biol Med 27: 951–965

Bryk R, Griffin P, Nathan C (2000) Peroxynitrite reductase activity of bacterial peroxiredoxins. Nature 407: 211–215

Budanov AV, Sablina AA, Feinstein E, Koonin EV, Chumakov PM (2004) Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. Science 304: 596–600

Carballal S, Radi R, Kirk MC, Barnes S, Freeman BA, Alvarez B (2003) Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite. Biochemistry 42: 9906–9914

Carter DC, He XM (1990) Structure of human serum albumin. Science 249: 302–303

Carter DC, He XM, Munson SH, Twigg PD, Gernert KM, Broom MB, Miller TY (1989) Three-dimensional structure of human serum albumin. Science 244: 1195–1198

Cha MK, Kim IH (2006) Disulfide between Cys392 and Cys438 of human serum albumin is redox-active, which is responsible for the thioredoxin-supported lipid peroxidase activity. Arch Biochem Biophys 445: 19–25

Claiborne A, Mallett TC, Yeh JI, Luba J, Parsonage D (2001) Structural, redox, and mechanistic parameters for cysteine-sulfenic acid function in catalysis and regulation. Adv Protein Chem 58: 215–276

Claiborne A, Miller H, Parsonage D, Ross RP (1993) Protein-sulfenic acid stabilization and function in enzyme catalysis and gene regulation. Faseb J 7: 1483–1490

Claiborne A, Yeh JI, Mallett TC, Luba J, Crane EJ 3rd, Charrier V, Parsonage D (1999) Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. Biochemistry 38: 15407–15416

DeLano WL (2002) The PyMOL Molecular Graphics System. *DeLano Scientific*, San Carlos, CA, USA. http://www.pymol.org

DeMaster EG, Quast BJ, Redfern B, Nagasawa HT (1995) Reaction of nitric oxide with the free sulfhydryl group of human serum albumin yields a sulfenic acid and nitrous oxide. Biochemistry 34: 11494–11499

Denu JM, Tanner KG (1998) Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. Biochemistry 37: 5633–5642

Di Simplicio P, Frosali S, Priora R, Summa D, Cherubini Di Simplicio F, Di Giuseppe D, Di Stefano A (2005) Biochemical and biological aspects of protein thiolation in cells and plasma. Antioxid Redox Signal 7: 951–963

Ellis HR, Poole LB (1997a) Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the AhpC component of alkyl hydroperoxide reductase. Biochemistry 36: 15013–15018

Ellis HR, Poole LB (1997b) Roles for the two cysteine residues of AhpC in catalysis of peroxide reduction by alkyl hydroperoxide reductase from Salmonella typhimurium. Biochemistry 36: 13349–13356

Era S, Hamaguchi T, Sogami M, Kuwata K, Suzuki E, Miura K, Kawai K, Kitazawa Y, Okabe H, Noma A et al. (1988) Further studies on the resolution of human mercapt- and nonmercaptalbumin and on human serum albumin in the elderly by high-performance liquid chromatography. Int J Pept Protein Res 31: 435–442

Era S, Kuwata K, Imai H, Nakamura K, Hayashi T, Sogami M (1995) Age-related change in redox state of human serum albumin. Biochim Biophys Acta 1247: 12–16

Finkel T (2000) Redox-dependent signal transduction. FEBS Lett 476: 52–54

Gatti RM, Radi R, Augusto O (1994) Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical. FEBS Lett 348: 287–290

Giles GI, Jacob C (2002) Reactive sulfur species: an emerging concept in oxidative stress. Biol Chem 383: 375–388

Halliwell B (1988) Albumin an important extracellular antioxidant? Biochem Pharmacol 37: 569–571

Halliwell B, Gutteridge JM (1990) The antioxidants of human extracellular fluids. Arch Biochem Biophys 280: 1–8

Hansen JM, Go YM, Jones DP (2006a) Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. Annu Rev Pharmacol Toxicol 46: 215–234

Hansen JM, Zhang H, Jones DP (2006b) Differential oxidation of thioredoxin-1, thioredoxin-2, and glutathione by metal ions. Free Radic Biol Med 40: 138–145

He XM, Carter DC (1992) Atomic structure and chemistry of human serum albumin. Nature 358: 209–215

Imai H, Hayashi T, Negawa T, Nakamura K, Tomida M, Koda K, Tajima T, Koda Y, Suda K, Era S (2002) Strenuous exercise-induced change in redox state of human serum albumin during intensive kendo training. Jpn J Physiol 52: 135–140

Inayama T, Kumagai Y, Sakane M, Saito M, Matsuda M (1996) Plasma protein-bound sulfhydryl group oxidation in humans following a full marathon race. Life Sci 59: 573–578

Janatova J, Fuller JK, Hunter MJ (1968) The heterogeneity of bovine albumin with respect to sulfhydryl and dimer content. J Biol Chem 243: 3612–3622

Kawai K, Yoh M, Hayashi T, Imai H, Negawa T, Tomida M, Sogami M, Era S (2001) Effect of diabetic retinopathy on redox state of aqueous humor and serum albumin in patients with senile cataract. Tokai J Exp Clin Med 26: 93–99

Keire DA, Strauss E, Guo W, Noszal B, Rabenstein DL (1992) Kinetics and equilibria of thiol/disulfide interchange reactions of selected biological thiols and related molecules with oxidized glutathione. J Org Chem 57: 123–127

Kice JL, Cleveland JP (1973) Nucleophilic substitution reactions involving sulfenic acids and sulfenyl derivatives. Nucleophile- and acid-catalyzed oxygen-18 exchange of phenyl benzenethiolsulfinate. J Amer Chem Soc 95: 104–109

Koppenol WH, Moreno JJ, Pryor WA, Ischiropoulos H, Beckman JS (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chem Res Toxicol 5: 834–842

Kratochwil NA, Ivanov AI, Patriarca M, Parkinson JA, Gouldsworthy AM, Murdoch P, Sadler PJ (1999) Surprising reactions of iodo Pt(IV) and Pt(II) complexes with human albumin: detection of Cys34 sulfenic acid. J Am Chem Soc 121: 8193–8203

Leto S, Yiengst MJ, Barrows CH Jr (1970) The effect of age and protein deprivation on the sulfhydryl content of serum albumin. J Gerontol 25: 4–8

Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS (2001) A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. Nature 410: 490–494

Mansoor MA, Svardal AM, Ueland PM (1992) Determination of the *in vivo* redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. Anal Biochem 200: 218–229

Moriarty-Craige SE, Jones DP (2004) Extracellular thiols and thiol/disulfide redox in metabolism. Annu Rev Nutr 24: 481–509

Noel JK, Hunter MJ (1972) Bovine mercaptalbumin and non-mercaptalbumin monomers. Interconversions and structural differences. J Biol Chem 247: 7391–7406

Paget MS, Buttner MJ (2003) Thiol-based regulatory switches. Annu Rev Genet 37: 91–121

Peshenko IV, Shichi H (2001) Oxidation of active center cysteine of bovine 1-Cys peroxiredoxin to the cysteine sulfenic acid form by peroxide and peroxynitrite. Free Radic Biol Med 31: 292–303

Peters T (1996) All about albumin: biochemistry, genetics and medical applications. Academic Press, San Diego

Poole LB, Zeng BB, Knaggs SA, Yakubu M, King SB (2005) Synthesis of chemical probes to map sulfenic acid modifications on proteins. Bioconjug Chem 16: 1624–1628

Quijano C, Alvarez B, Gatti RM, Augusto O, Radi R (1997) Pathways of peroxynitrite oxidation of thiol groups. Biochem J 322: 167–173

Radi R, Beckman JS, Bush KM, Freeman BA (1991a) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem 266: 4244–4250

Radi R, Bush KM, Cosgrove TP, Freeman BA (1991b) Reaction of xanthine oxidase-derived oxidants with lipid and protein of human plasma. Arch Biochem Biophys 286: 117–125

Raftery MJ, Yang Z, Valenzuela SM, Geczy CL (2001) Novel intra- and inter-molecular sulfinamide bonds in S100A8 produced by hypochlorite oxidation. J Biol Chem 276: 33393–33401

Sahoo R, Dutta T, Das A, Sinha Ray S, Sengupta R, Ghosh S (2006) Effect of nitrosative stress on Schizosaccharomyces pombe: inactivation of glutathione reductase by peroxynitrite. Free Radic Biol Med 40: 625–631

Salmeen A, Andersen JN, Myers MP, Meng TC, Hinks JA, Tonks NK, Barford D (2003) Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. Nature 423: 769–773

Salmeen A, Barford D (2005) Functions and mechanisms of redox regulation of cysteine-based phosphatases. Antioxid Redox Signal 7: 560–577

Saurin AT, Neubert H, Brennan JP, Eaton P (2004) Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. Proc Natl Acad Sci USA 101: 17982–17987

Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 30: 1191–1212

Schnitzer JE, Sung A, Horvat R, Bravo J (1992) Preferential interaction of albumin-binding proteins, gp30 and gp18, with conformationally modified albumins. Presence in many cells and tissues with a possible role in catabolism. J Biol Chem 267: 24544–24553

Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, Loscalzo J (1992) S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc Natl Acad Sci USA 89: 444–448

Stewart AJ, Blindauer CA, Berezenko S, Sleep D, Tooth D, Sadler PJ (2005) Role of Tyr84 in controlling the reactivity of Cys34 of human albumin. Febs J 272: 353–362

Sugio S, Kashima A, Mochizuki S, Noda M, Kobayashi K (1999) Crystal structure of human serum albumin at 2.5 A resolution. Protein Eng 12: 439–446

Surdhar PS, Armstrong DA (1986) Redox potentials of some sulfur-containing radicals. J Phys Chem 90: 5915–5917

Takakura K, Beckman JS, MacMillan-Crow LA, Crow JP (1999) Rapid and irreversible inactivation of protein tyrosine phosphatases PTP1B, CD45, and LAR by peroxynitrite. Arch Biochem Biophys 369: 197–207

Tomida M, Ishimaru J, Hayashi T, Nakamura K, Murayama K, Era S (2003) The redox states of serum and synovial fluid of patients with temporomandibular joint disorders. Jpn J Physiol 53: 351–355

Torchinsky YM (1981) Sulfur in proteins. Pergamon Press Ltd, Oxford

Trujillo M, Budde H, Pineyro MD, Stehr M, Robello C, Flohe L, Radi R (2004) Trypanosoma brucei and Trypanosoma cruzi tryparedoxin peroxidases catalytically detoxify peroxynitrite via oxidation of fast reacting thiols. J Biol Chem 279: 34175–34182

Trujillo M, Radi R (2002) Peroxynitrite reaction with the reduced and the oxidized forms of lipoic acid: new insights into the reaction of peroxynitrite with thiols. Arch Biochem Biophys 397: 91–98

van der Vliet A, Hoen PA, Wong PS, Bast A, Cross CE (1998) Formation of S-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide. J Biol Chem 273: 30255–30262

van Montfort RL, Congreve M, Tisi D, Carr R, Jhoti H (2003) Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. Nature 423: 773–777

Wilson JM, Wu D, Motiu-DeGrood R, Hupe DJ (1980) A spectrophotometric method for studying the rates of reaction of disulfides with protein thiol groups applied to bovine serum albumin. J Am Chem Soc 102: 359–363

Wink DA, Nims RW, Darbyshire JF, Christodoulou D, Hanbauer I, Cox GW, Laval F, Laval J, Cook JA, Krishna MC et al. (1994) Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solutions at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O_2 reaction. Chem Res Toxicol 7: 519–525

Winterbourn CC, Metodiewa D (1999) Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. Free Radic Biol Med 27: 322–328

Authors' address: Rafael Radi, Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, Avda. Gral. Flores 2125, 11800 Montevideo, Uruguay,
Fax: +598-2-9249563, E-mail: rradi@fmed.edu.uy