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Thiol disulfide exchange reactions in human serum albumin: the apparent paradox of the redox transitions of Cys_{34}

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Running title: *Redox transition of Cys_{34} in human serum albumin*

Abbreviations: HSA, human serum albumin; GSH, glutathione (reduced form); GSSG, oxidized glutathione; Cys-SH, cysteine; CysS-SCys, cystine; CysGly-SH, cysteinylglycine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NBD-Cl, 4-chloro-7-nitrobenzofurazan

Keywords: Cys_{34} of human serum albumin; disulfide stability in albumin; redox transitions in albumin; protein disulfides; protein thiols; dialytic therapy; oxidized albumin; oxidative stress; effective molarity.

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ABSTRACT

Human serum albumin (HSA) is characterized by 17 disulfides and by only one un-paired cysteine (Cys_{34}), which can be free in the reduced albumin or linked as a mixed disulfide with cysteine, or in minor amount with other natural thiols, in the oxidized albumin. In healthy subjects, the level of the oxidized form is about 35%, but it rises up to 70% after oxidative insults or in patients with kidney diseases. Oxidized albumin is therefore considered a short-term biomarker of oxidative stress as its level may increase or decrease under appropriate redox inputs in discrete temporal spans. This paper defines, for the first time, the kinetic properties of reduced and oxidized Cys_{34} of HSA in their reaction with natural disulfides and thiols. Kinetic constants support the evidence that the Cys_{34} redox oscillations observed *in vivo* are mainly due to the interaction with cysteine and cystine without the involvement of any enzymatic support. This study gives also a plausible explanation for the absence of involvement of the 17 disulfides naturally present in HSA in these redox transitions. This inert behavior toward cysteine is marginally due to solvent accessibility or flexibility factors of these bonds but mainly to their strong thermodynamic stability, which is caused essentially by a

proximity effect. A similar mechanism is likely at play in the many proteins that maintain disulfide bridges in a reducing medium like the cytosol.

Introduction

Serum is characterized by an oxidizing environment and in that milieu most of the natural low mass thiol compounds are present as disulfides, i.e. cystine, homocystine, cystamine and GSSG [1]. Some of these sulfur compounds are also present as mixed disulfides with protein cysteins. Usually the reduced forms of these compounds represent no more than 3-5% of their total concentration [1]. One curious exception is represented by Cys_{34} of HSA, which is the most abundant protein in plasma, reaching the concentration of 0.6-0.8 mM [2]. HSA comprises 17 disulfide bonds and one free cysteine, i.e. Cys_{34} [2]. In healthy subjects, this residue is mainly present in a reduced form (about 70%) (HSA_{red}) while 25-30% is found as a reversible oxidized form, i.e. as mixed disulfide with cysteine and in minor amounts with cysteinylglycine, homocysteine and GSH (HSA_{ox}) [3,4]. Irreversible oxidized forms, like sulfinate and sulfonate, are also found in very small amounts (3-4%) [4]. Thus, about 0.4 mM of a protein –SH group is normally present in serum, representing the strongest reducing power inside this fluid [5]. Under pathologic conditions, like kidney or liver diseases, the level of oxidized albumin may increase up to 70% [6-11]. High levels have been also observed in athletes after extreme exercises [12]. In this context, details on the mechanism and kinetics of these redox changes are lacking. In particular, some questions are still to be clarified: for example, an evident but unexplained paradox is the presence of 70-80% reduced Cys_{34} in albumin co-existing with 70-80% of oxidized low molecular mass thiols presented as disulfides. It is also unclear why cysteine is the compound that mainly forms the oxidized mixed disulfide with Cys_{34}. It should be also clarified if the redox transitions involving Cys_{34} and cysteine/cystine are only due to the intrinsic reactivity of these reagents or kinetically favored by some form of enzyme catalysis Finally, is only Cys_{34} involved in a redox transition of albumin while the entire pool of its 17 disulfides remains unperturbed in their oxidized form? It has been recently found that many of these structural cysteines in the reduced form are hyper-reactive toward many reagents, including disulfides [13], so it may be also interesting to verify their reactivity toward thiols when present in the oxidized form.

Both the equilibrium and rate constants of these redox reactions are unknown [1]. This ultimately limits our understading of the overall redox scenario involving albumin and the effect that these redox transitions may have in the function of this and related proteins.

Motivated by the above considerations we obtained here a detailed kinetic pathway of the Cys34 redox interplay in HSA. By doing so we uncovered surprising and unknown properties of the sulfur chemistry of the most abundant and studied protein of the human serum.

Results

Changes of oxidized serum albumin in kidney dialyzed patients

It is well known that dialyzed patients display a high level of oxidized albumin, but conflicting results have been reported about its variation before and after the dialysis treatment. For example, Wlodek et al. [14] reported a very similar value for the protein-bound cysteine (i.e. oxidized albumin) before and after dialysis (0.24 mM and 0.25 mM) while Terawaki et al. [15] observed large variations (from 55% to 33% respectively). We examined 20 patients under dialytic treatment obtaining 59 ± 3% and 42 ± 2% of oxidized albumin before and after dialysis, while 39 ± 3% is found in healthy subjects (Fig. 1A). By considering that about 90% the oxidized albumin is represented as the mixed disulfide Cys_{34}S-SCys and that the average concentration of albumin is 0.7 mM [4], it results that about 0.1 mM of cysteine is released from the protein to the plasma during about three hours of dialysis. At present, the reducing compound that preferentially acts on the Cys_{34} mixed disulfide is still unknown. Conversely, re-oxidation of albumin is a slower event, occurring within 24-36 h and possibly involving a direct interaction with the free cystine. This example further illustrates the clinical and diagnostic importance of the redox equilibria of HSA. To clarify all the actors involved in these redox events and the possible occurrence of some enzymatic assistance, it seems appropriate to characterize the kinetic propensity of Cys_{34} (either in its reduced or in its mixed disulfide form) to react with all natural thiols and disulfides present in a normal serum.

Interaction between Cys_{34}S-SCys and different natural thiols

Kinetic constants of Cys_{34} reduction by different thiol compounds have been obtained by observing the time-dependent increase of the Cys_{34} concentration or, alternatively, the decrease of the thiol used as reducing agent (see Materials and Methods).

The most efficient reducing compounds present in serum are cysteine and cysteinylglicine and the least efficient is GSH (Table 1). Except for cysteamine, which is not present in serum and whose curious hyper-reactivity will be discussed below, a good correlation has been found between the pK_a of the thiol compounds and the second order rate constants indicating that these kinetic differences can be due mainly to the different deprotonation of the sulfhydryl group (Fig. 1B). Conversely, no correlation was found for the pH independent *k* values (i.e. *k* at full deprotonation)

vs pK_a of all these thiols (data not shown). This result is probably due to the limited pK_a range (from 8.43 to 9.2) that does not allow the visualization of the predicted decrease of nucleofilicity by increasing the thiol acidity [16,17]. After reaction with albumin, the decrease of the thiol compound is stoichiometric to the amount of the newly formed reduced Cys_{34}, suggesting that each thiol compound only interacts with the mixed disulfide of Cys_{34} without the involvement of any of the 17 protein disulfide bridges (not shown). **Otherwise, the decreased amount of the free thiol** after reaction **must exceed the amount of the newly formed reduced Cys34. This agrees well with the never described occurrence, in normal sera, of mixed disulfides between natural thiols and albumin protein cysteines usually involved in intramolecular disulfide bonds. Only more drastic interaction conditions will reveal very small amounts of such unusual mixed disulfides (see below).**

By plotting the pseudo-first order constants for the reduction vs. the concentration of the thiol, a strict linearity indicates the absence of a possible fast pre-complex formation during the reduction pathway (Fig. 2) (in this case, a saturation behavior should be observed as in the case of enzymatic kinetics). On the basis of the *in vivo* concentrations of these thiol compounds in normal human serum (Fig. 3A) and of their kinetic ability to reduce the mixed disulfide of Cys_{34} (Table 1), an estimated "*in vivo kinetic reducing power*" for each thiol can be calculated as $k_{red} \times [RSH]_{\text{in vivo}}$, indicating again cysteine as the more active compound (Fig. 3B).

Oxidation of reduced Cys_{34} by natural serum disulfides

The reverse reaction, i.e. the oxidation of Cys_{34} to give mixed disulfides may occur by following two different pathways. The first one is the direct reaction with natural low-molecular weight disulfides like cystine, homocystine, GSSG etc. Kinetics of all these reactions can be easily studied by following the continuous or end point procedures described under the Materials and Methods section. Beside cystamine (not present in serum), cystine and cystinylglycine are the most active disulfides, with kinetic constants (0.6 $M^{-1}s^{-1}$) about ten times lower than the one found for cysteine in the reverse reaction (6.6 $M^{-1}s^{-1}$) (Table 1 and 2). A good correlation has been found between log *k* and the pK_a of the leaving thiols (Fig. 1C). Also in this case, on the basis of the concentrations of the different disulfides *in vivo* (Fig 3A) and of their kinetic efficiency (Table 2), the "*in vivo kinetic oxidizing powers*" i.e. $k_{ox} \times [RSSR]_{\text{in-vivo}}$, indicate cystine as the more active disulfide (Fig. 3B). Accordingly, this protein mixed disulfide is the most abundant in a normal human plasma (Fig. 3A). It has been found that binding of fatty acids to albumin (about 5:1 mole:mole) modulates the reactivity of Cys_{34}, inducing structural modification near Cys_{34} and also lowering its pK_a [18]. We re-calculated the kinetic constants for the redox reactions involving Cys_{34} for a non-purified

albumin i.e. in the presence of its original serum. However, circulating HSA in healthy subjects carries only 0.3-1 fatty acids per albumin molecule [19]. Data reported in Table 1 and 2 indicate only small changes of the kinetic constants for both oxidative and reductive reactions involving natural thiols and disulfides.

Oxidation of reduced Cys_{34} via sulfenic acid

One alternative pathway for the oxidation of Cys_{34} to form a mixed disulfide is the one occurring under severe oxidative stress characterized by production of inorganic or organic peroxides. H_2O_2 is the most common inorganic peroxide. This compound reacts with Cys_{34} at a rate similar to the one observed with oxidized thiol compounds (k = 2.3 $M^{-1}s^{-1}$) [20] and producing a stabilized sulfenic acid [21]. This first oxidized product interacts with free reduced cysteine at very high rate (k = 21 $M^{-1}s^{-1}$) [22] giving the mixed disulfide HSA-Cys_{34}S-SCys. It is reasonable that this oxidative pathway should be only operative in case of severe oxidizing conditions. Interestingly, cysteinylglycine which *in vivo* is about one fourth of the cysteine concentration [1], reacts with Cys_{34}-sulfenic acid at higher rate than cysteine, so the *in vivo oxidizing power* is similar to the one shown by cysteine (Fig 3B). As it will be discussed below, this property will help to distinguish if the oxidized albumin comes from a simple reaction with cystine or via sulfenic acid.

By assuming the levels of all thiols and disulfides *in vivo* as constant, and the values for the *in vivo reducing and oxidizing powers* (Fig. 3), the predicted percentual values of oxidized and reduced albumin are in good agreement with those found *in vivo* (Fig. 4).

The 17 natural disulfides are not involved in the redox interplay of serum albumin

The involvement of some of the 17 natural disulfides in redox changes of albumin has been never described or verified in the plasma of healthy subjects. Only in a recent study the presence of albumin with mixed disulfides involving other protein cysteines like Cys_{90}, and Cys_{112} in hyper-lipidemic patients was discovered [23]; indeed, even partial disruption of its disulfide bridges could represent a dramatic event as such albumin undergoes deleterious aggregation and amyloid polymerization [24]. It is thus interesting to verify whether the most active low molecular mass reducing compound in serum i.e. free cysteine really interacts exclusively with the mixed disulfide of Cys_{34} in spite of the presence of 17 times more abundant protein disulfides. We tested the ability of this amino acid to reduce the protein disulfides. Using albumin concentration similar to the one recovered in healthy subjects (0.7 mM) and high amount of free cysteine (1.5 mM) (about 150 times higher than that found in plasma) we observed that only a very low disulfide reduction occurred (about 0.5%). The reaction reached equilibrium in a few minutes and the very low amount

of reduced protein disulfides remains unchanged even after 30 min incubation at 37 °C. Due to the very low product concentration at equilibrium, kinetic constants cannot be easily calculated but the corresponding equilibrium constant was evaluated as K = 3.3 M^{-1}. Considering that the normal cysteine concentration in serum is about 10 µM, i.e 150 times lower than the value used in our experiment, our result predicts that the reduction of protein disulfides *in vivo* is always negligible.

Structural accessibility and flexibility of all cysteines in albumin do not explain the exclusive involvement of Cys_{34} in its redox transitions

The absence of a quantitative reduction of some of the 17 natural disulfides of albumin when the protein is incubated with high concentration of cysteine and, on the contrary, the exclusive involvement of the mixed disulfide of Cys_{34} could be due to a scarce accessibility or flexibility of the disulfide bridges in the protein. However, solvent accessibility and B-factors clearly indicate that at least 8-10 cysteines display similar or higher solvent accessibility and flexibility than Cys_{34} (Fig. 5). In particular, Cys_{514}, Cys_{558} and Cys_{564} at the C-terminal segment of albumin display much higher accessibility and flexibility than Cys_{34}. Thus, the most plausible cause of the exclusive involvement of Cys_{34} resides in the very strong thermodynamic stabilization of all its disulfides. This may be rationalized by assuming that a first interaction of cysteine with some protein disulfide may actually occur, but the reverse reaction between the HSA-CysS-SCys mixed disulfide and the neighboring cysteine is much faster.

About the reactivity of Cys_{34}

By comparing the kinetic constant of Cys_{34} in its reaction with free cystine (0.6 $M^{-1}s^{-1}$) (similar for albumin in serum) with the one of GSH (which mimics well a cysteine inserted in a polypeptide chain) with cystine (0.7 $M^{-1}s^{-1}$) [13], a very similar kinetic propensity is observed. Thus, no hyper-reactivity may be ascribed for this residue, at least for its reaction with neutral disulfides. On the contrary, the extraordinary reactivity of Cys_{34} with cystamine is well known [25], and we also observed a similar high reactivity when its reduced form, i.e. cysteamine, reacts with the mixed disulfide Cys_{34}S-SCys (Table 1). This hyper-reactivity is mainly determined by electrostatic properties of the protein environment around the sulfur atom of Cys_{34}. A computational prediction of pK_a using PropKa [26] gave a value of 11.0. This value is 2 units higher than a normal protein cysteine, due to the presence of negative charges near this residue (particularly Asp_{38}). However, our experimental determination of this pK_a using DTNB as thiol reagent yielded a normal value of 9.0 for the purified HSA and 8.8 in the presence of human plasma (data not shown). Other studies, using different methodologies and other experimental conditions (buffers, temperature etc.) found

even lower values [27]. This discrepancy between experimental (pK_a 8-9) and the computational values (pK_a 11.0) may be explained by assuming that only in the crystal state the free carboxylate of Asp_{38} is near enough to modify the acid-base property of Cys_{34}. The structure may be altered in solution, were the carboxylate and Cys_{34} could be more distant. In any case, the effect of this negative charge may be still crucial to drive in cationic reagents (i.e. cystamine and cysteamine) towards Cys_{34} accelerating the reaction. The overall negative charge of HSA could also play an additional role in this context.

Discussion

It is well accepted that inside the cell a reducing medium is prevalent, while extracellular fluids display a more oxidized environment. Thus, the presence of a more abundant level of reduced albumin (about 60-70%) in healthy subjects [3] in equilibrium with relevant amounts of oxidized forms of low molecular weight thiols like cystine, cystinylglycine and GSSG [1] represents a sort of thermodynamic paradox that can be reasonably explained only on the basis of the kinetic barriers described in the present study. A preliminary question must be first clarified i.e. if the small concentration of low mass thiols drives the redox transition of albumin, or vice versa. Albumin in plasma has a long half-life of about 20 days [28], while for small natural thiols the half-life is only a few min [29]. For example, cysteine flux in human plasma is 20 μM/min [30] and being 60 μM the total concentration of the free cysteine/cystine in this compartment, a reasonable turnover may occur within only a few minutes. Thus it is evident that low molecular weight free thiols drive the redox status of albumin. Conversely, this protein appears to act as a redox buffer avoiding that the CysSH/CysS-SCys ratio undergoes relevant changes. By assuming that the flux of cysteine/cystine in serum is not rate limiting, an examination of the second-order kinetic constants for the reaction of Cys_{34} and its more abundant oxidized form (i.e. its mixed disulfide with cysteine) with natural disulfides and thiols likely identifies the free cysteine/cystine couple as the major actor for the redox transitions of albumin. However cysteinylglycine, showing similar kinetic constants, must be also accounted.

By considering the steady-state concentrations of each thiol compound *in vivo*, the calculated "*kinetic reducing and oxidizing powers"* of each redox couple, i.e. cysteine/cystine, GSH/GSSG, etc. are useful tools to rationalize this complex scenario, confirming the cysteine/cystine couple as the prominent thiol/disulfide system in determining the redox status of Cys_{34}. Cysteinylglycine appears less important, due to its lower concentration in serum. These quantitative data and

conclusions fully explain the presence *in vivo* of Cys_{34}S-SCys as the most abundant mixed disulfide involving Cys_{34} in healthy subjects.

A more accurate examination of the kinetic constants reported in Table 1 and 2 allows further characterization of the redox scenario present in serum. In healthy subjects the steady-state concentration of the most represented high and low mass thiols is well known, i.e. HSA_{ox} = 0.185 mM, HSA_{red} = 0.42 mM, CysSH = 10 μM and CysS-SCys = 50 μM (average values from a few studies) [1]. Thus, the apparent equilibrium constant for the redox reaction:

HSA_{ox} + CysSH ⇆ HSA_{red} + CysS-SCys

is:

K_{eq}=[HSA_{red}][CysS-SCys]/[HSA_{ox}][CysSH]

0.42 mM × 0.05 mM / 0.185 mM × 0.01 mM = 11.4.

This value is very close to the ratio of the two kinetic constants reported in Tables 1 and 2, corresponding to the following velocities:

$v_{ox} = k_{ox}$[HSA_{red}][CysS-SCys] and

$v_{red} = k_{red}$[HSA_{ox}][CysSH]

i.e. k_{red}/k_{ox} = 11

a value that confirms the accuracy of our kinetic study. Furthermore, using these reliable kinetic constants, and assuming unchanged the total free cysteine level (CysSH + CyS-SCys), it is easy to predict that, in serum, a 10% increase in oxidized cysteine is enough to cause an increased concentration of HSA_{ox} at equilibrium similar to the one found in nephropathic patients before dialysis (65%, i.e. 0.42 mM HSA_{ox} and 0.18 mM HSA_{red}). In fact: [0.18 mM][0.054 mM]/[0.42 mM][0.002 mM] = 11.

As demonstrated by Alvarez and co-workers the reactivity of Cys_{34} of HSA is modulated by the binding of fatty acids as observed when the complex fatty acid:albumin is 5:1 [18]. Our kinetic study on human albumin in the presence of its normal plasma signaled only slight variations of the kinetic constants in the reaction with thiol and disulfides (see Table 1 and 2), a reasonable result given that only 0.3-1 mole of fatty acid is bound to albumin in a normal serum [19].

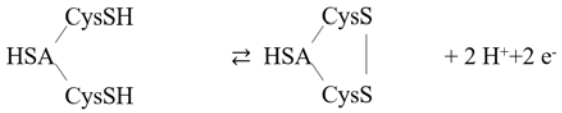
On the basis of all kinetic data reported in this study and the prominent role of the cysteine/cystine couple, a rationale scheme of the Cys_{34} redox interplay is reported below (Fig. 6).

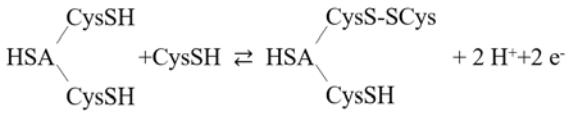
Our kinetic data and the *in vivo kinetic powers* also suggest that a relevant involvement of hydrogen peroxide in the redox transition of Cys_{34} (as it must occur during a strong oxidative stress) would be signaled by a conspicuous concentration of the mixed disulfide Cys_{34}S-SCysGly, always assuming that the flux of this compound in serum is not rate-limiting. The low amount of this mixed disulfide

in normal serum (only about 10% compared to that involving cysteine [1]) is a signal that the direct interaction of Cys_{34} with cystine predominates and indicates the absence of strong oxidative stress.

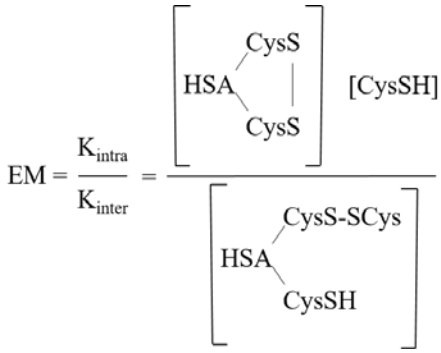
A second interesting question regards the exclusive involvement of Cys_{34} in the redox transition of albumin and the apparent absence of interaction of natural thiols with the seventeen disulfides of this protein. Our results indicate that the redox inertia is confirmed by an experimental equilibrium constant (K_{eq} = 3.3 M^{-1}) that suggests a negligible reduction even at very high levels of cysteine. This high stability cannot be ascribed to a scarce accessibility nor to a high flexibility of the protein disulfides, because some of them are more exposed and flexible than Cys_{34} (Fig. 5). Rather, it is likely due to the "entropic factor" known as "effective concentration", a parameter evoked to explain both the acceleration of reactions occurring in the active site of enzymes and the redox stability of protein disulfides [31]. Effective concentration, or effective molarity (EM) is defined as the ratio of the first-order rate constant of an intramolecular reaction involving two functional groups within the same molecular entity to the second-order rate constant of an analogous intermolecular elementary reaction. It can be similarly defined based on the ratio of the equilibrium constants for the intramolecular and intermolecular reactions. This ratio has the dimension of concentration, and indicates the concentration of the external reagent for which the intermolecular reaction becomes comparable to the intramolecular one [32].

In our case, the intramolecular reaction can be formally described as the formation of a disulfide bridge, while the intermolecular reaction corresponds to disulfide formation with an external thiol:

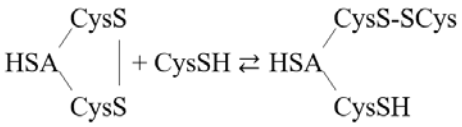




Therefore, the effective molarity corresponds to:



It is easy to see that the EM corresponds to the reciprocal value of the equilibrium constant of the following exchange reaction:



Based on the data reported above for this reaction, EM is therefore $1/(3.3\ M^{-1}) = 0.3$ M.

This value is orders of magnitude higher than the concentration of free low molecular weight thiols in plasma (Fig. 3A) and illustrates the prevalence of intramolecular disulfide formation with respect to the formation of mixed disulfides when an adjacent cysteine is present in the protein. At the same time, in the case of Cys_{34} no intramolecular reactive group is present, and therefore the intermolecular reaction takes place. We also note that an EM value of 0.3 M for the vicinal cysteines in albumin agrees with the near complete reduction of all disulfide bridges of this protein by stoichiometric amounts of dithithreitol [13], an intramolecular dithiol compound whose sulfhydryls show an EM of 600 M [29].

The "effective concentration" is a convincing tool which explains the paradoxical permanence of disulfides in proteins and enzymes residing in a reducing medium like the cytosol i.e. ribonuclease, phospholipase, aldolase etc. In a very recent study, it has been observed that only in the presence of pathological levels of fatty acids, cysteines different from Cys_{34} can form mixed disulfides with cysteine or homocysteine [23]. It is possible that the binding of multiple fatty acids to albumin induces a steric tension which turns away the two cysteines once the original bridge has been disrupted. Unfortunately, no structural evidence for this interpretation is today available.

In conclusion, this study clarifies some still obscure redox dynamics involving albumin and its peculiar Cys_{34} and fulfills a more solid base to use this protein as a biomarker for oxidative stress induced by pathological conditions or by over-training occurring in athletes.

Materials and methods

Chemicals and reagents

HSA was a SIGMA product. The product number is A3782. In this particular commercial stock HSA_{red} is 26% HSA_{ox} is 46% and HSA as irreversibly oxidized form is 17%, (10% dimer) as reported in [33]. The Cys_{34}-cysteine mixed disulfide was between 90/95% of the HSA_{ox} fraction, as measured by adopting the following procedure: HSA (0.03 mM) was reated with an excess of DTT (0.3 mM) in 0.1 M sodium borate buffer, pH 8.5. After 30 min the free cysteine was titrated by adding 1 mM of bromopyruvate. The photometric procedure for the determination of the cyclic product (lanthionine ketimine) is reported below. Cysteine, cystine, cystamine, GSH, oxidized glutathione, DTNB, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), H_2O_2, cysteamine,

homocysteine, cysteinylglycine and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification.

Healthy subjects and patients enrollment

The study protocol complied with the declaration of Helsinki and a written fully informed consent was provided by all patients and healthy subjects before enrollment into the study. The present research is approved by the Ethical Committee of Azienda Ospedaliera Universitaria, Policlinico "Tor Vergata". The study is an investigation on 20 healthy subjects and 20 uremic patients under hemodialysis. Blood samples from healthy subjects were collected from the anticubital vein into K-3-EDTA tubes. Blood samples from patients were collected from the arterial site of the vascular access before the dialysis and at the end (about three hours) of the dialysis session. Exclusion criteria in both healthy subjects and patients were a clinical history of hepatitis C and B, serum alanine aminotransferase and/or aspartate aminotransferase twice the upper limit of normal values, morbid obesity, rheumatological disorders, active cancer and pregnancy. Plasma was purified from whole blood samples and albumin level (g/dL) was determined by routine protocol according to the standard procedures in the Clinical Chemical Laboratories (Policlinico, University of Rome, Tor Vergata) by Dimension VISTA 1500 (Siemens Healthcare Diagnostics, Milan, Italy).

Quantitation of the reduced albumin

Cys_{34} in the reduced albumin reacts with DTNB at very low velocity [13,34] so its quantification in a short time was performed taking advantage by the very fast reaction of Cys_{34} with cystamine (see Table 1) [35]. The released cysteamine (stoichiometric to Cys_{34}) can be titrated within two min with DTNB (see below). $TNBS^{--}$ displays an ε_M = 14,100 $M^{-1}cm^{-1}$ at 412 nm. In a typical experiment 0.025 mM of serum albumin, diluted in 0.89 ml of 0.1 M potassium phosphate buffer, pH 8.0, were reacted with 1 mM cystamine ($R^1S\text{-}SR^1$) and 0.05 mM DTNB (1 ml final volume) at 25 °C. The absorbance at 412 nm was registered after two minutes.

(a) HSA-Cys_{34}SH + DTNB $\xrightarrow{slow}$ HSA-Cys_{34}S-STNB + $TNBS^{--}$

(b) HSA-Cys_{34}SH + cystamine ($R^1S\text{-}SR^1$) $\xrightarrow{fast}$ HSA-Cys_{34}S-SR^1 + cysteamine (R^1SH)

(c) cysteamine (R^1SH) + DTNB $\xrightarrow{fast}$ R^1S-STNB + $TNBS^{-}$

Kinetics of oxidation of Cys_{34} with natural disulfides

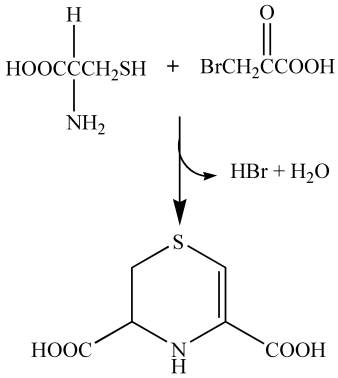
Kinetics were studied by reacting a solution of commercial HSA (36 μM containing 26% of reduced albumin) with disulfides i.e. cystine, homocystine, GSSG, cystinylglycine, etc. (1 mM) at

pH 7.4 and 37 °C. At fixed times, the released free thiol (cysteine, homocysteine, GSH, etc.) was measured on aliquots on the basis of their reaction with 0.1 mM DTNB. This quantitation is possible because the free thiol is stoichiometric to the Cys_{34} and it reacts with DTNB in a few seconds (k = 7000 $M^{-1}s^{-1}$), while the residual Cys_{34} reacts very slowly (k =13 $M^{-1}s^{-1}$) [22].

$HSA\text{-}Cys_{34}SH + RS\text{-}SR \longrightarrow HSA\text{-}Cys_{34}S\text{-}SR + RSH$

$RSH + DTNB \longrightarrow RS\text{-}STNB + H^+ + TNBS^-$ (yellow colored ion absorbing at 412 nm)

Alternatively, cysteine and GSH were quantified with more specific procedures: GSH was titrated using an enzymatic assay based on the conjugation of GSH with NBD-Cl catalyzed by glutathione transferase P1-1 [36]. The reaction was performed on 0.1 ml aliquots of the above described mixtures containing HSA and natural disulfides (withdrawn at fixed times) diluted in 0.9 ml of 0.1 M acetate buffer pH 5.0 containing 0.2 mM NBD-Cl and 1 unit of GSTP1-1. The end-point conjugate adduct GS-NBD (ε_M = 13,000 $M^{-1}cm^{-1}$ at 420 nm) is formed within 2 min and allows a precise quantitation of the free GSH produced in the mixture. Cysteine was titrated at fixed times by reacting aliquots of the original incubation mixture (HSA + variable amounts of cystine) with 0.1 mM bromopyruvate. The alkylation product is a ketimine ring (lanthionine ketimine) which absorbs at 296 nm (ε_M = 6,200 $M^{-1}cm^{-1}$) [37]. The procedure is represented below:



Lanthionine ketimine

A supplementary procedure was also adopted to evaluate the oxidation rate of Cys_{34} with natural disulfides based on the spectrophotometric method in continuous proposed by Wilson et al. [38].

Kinetics of reduction of the mixed disulfide involving Cys_{34} by natural thiols

Kinetics were studied by reacting commercial HSA (10 μM) (containing 46% of oxidized protein as mixed disulfide) [33] with variable amounts (from 18 μM to 110 μM) of natural thiols i.e. cysteine, homocysteine, GSH, cysteinylglycine, etc. at pH 7.4 and at 37 °C. At fixed times the decrease of free thiol concentration (due to the reduction of Cys_{34} mixed disulfide) was assayed by reacting aliquots of the mixture with DTNB (0.1 mM) at pH 7.4. Under these conditions, residual free thiol reacts within a few seconds with DTNB, while Cys_{34} reacts very slowly making easy the

determination of the reduction event. The pH of the same cuvette was then raised to pH 9.0 to titrate **the total reduced Cys34 after thirty minutes of additional incubation. By subtracting the amount of Cys34 present in the albumin before reaction with the natural thiols, the newly formed reduced Cys34 can be easily determined.**

Reduction of the structural protein disulfides by cysteine

0.7 mM HSA was incubated with 1.5 mM cysteine at pH 7.4 and EDTA at 37 °C. At fixed times the reaction mixture was passed through a Sephadex G-25 column (0.8 x 16 cm) to eliminate the excess of cysteine, and the amount of reduced disulfides evaluated on the basis of their hyper-reactivity with DTNB [13]. Briefly, the eluate from the Sephadex column containing 0.16 mM HSA was reacted at pH 6 with 0.1 mM DTNB. The reaction was followed for 2 min at 412 nm. Under these conditions only reduced cysteines coming from the natural disulfides reacts fast and quantitatively with DTNB while Cys_{34} does not react at all with DTNB [13].

Determination of pK_a of Cys_{34}

Experiments were performed by reacting 18 µM of reduced purified albumin with 0.1 mM DTNB in 0.02 M Britton-Robinson buffer at different pH values from 7.4 to 10 (1 ml final volume). Spectrophotometric measurements were made at 412 nm by following in continuous the release of $TNBS^{--}$. The same procedure was used to test the human albumin in the presence of plasma (diluted 1:20).

Computational study

The accessible surface area of the 35 cysteine residues of HSA were calculated using the algorithm POPS* v3.1 as implemented in the server [39]. Average B-factors were derived from the sulfur atom of the 35 cysteines and all atoms surrounded Cys thiol group in a radius of 5 Å. The atoms were identified by zone selection and structural analysis, attribute calculation tools implemented on UCSF Chimera software [40]. The crystallographic structure for computational studies was the PDB entry 1AO6 [41]. The pK_a calculation of Cys_{34} from pdb id: 1AO6 was derived by means of PropKa software [26].

Data analysis

Data are reported as means ± standard deviation (SD). The statistical calculation was made by GraphPad calc (on-line) and graphical representations were performed by GraphPad Prism software (La Jolla, CA, USA).

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Author contributions: A.B, conducted most of the experiments and analyzed the results. G.C. conducted the experiments. L.S. collaborated to logic development of the paper and analyzed the data. R.M. collected plasma samples and coordinated the study among clinical units.
G.R. wrote the paper and coordinated the entire study.

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Figure legends

Fig. 1. Changes of oxidized albumin due to dialysis and influence of the acidity of natural thiols on the redox rate constants of Cys_{34}. **(**A**)** Level of total oxidized albumin in healthy subjects (green bar) and in dialyzed patients before and after the dialytic session (black and red bars, respectively). Drawing and determinations of oxidized albumin were made as reported under Materials and Methods. Error bars are SD. (B) Correlation of the reduction rate constants of the Cys_{34} mixed disulfide by natural thiols (from Table 1) vs the pK_a of their sulfhydryl group. pK_a values: GSH = 9.2 [42]; Cysteine = 8.53 [42]; Cysteamine = 8.35 [42]; Homocysteine = 8.87 [43]; Cysteinylglycine = 8.43 [44]. (C) correlation of the oxidation rate constants of Cys_{34} by natural disulfides vs the pK_a of their corresponding sulfhydryl group. In panel (B) and (C) error bars (SD) fall within the symbols.

Fig. 2. Dependence of the reduction rates of oxidized albumin on low mass thiol concentrations. Rate of reduction was determined by incubating commercial HSA with variable amounts of natural thiols at pH 7.4, 37 °C, as described under Materials and Methods. (A) Homocysteine. (B) Cysteine. (C) Cysteinylglycine. Error bars are SD calculated from three independent experiments.

Fig. 3. *In vivo* levels and "*in vivo reducing and oxidizing powers"* of natural thiols and disulfides. (A) *In vivo* levels of low molecular mass thiols as reduced, oxidized and like mixed disulfides bound to protein. Data are averages ± SD of the levels reported by several studies (see Ref. 1). (B) "*In vivo reducing and oxidizing powers*" have been calculated by multiplying each kinetic constant (Table 1 and 2) for the corresponding *in vivo* plasma average concentration of natural thiols and disulfides reported in panel (A) Red bars are taken from Ref. 21.

Fig. 4. Experimental and theoretical percent values of oxidized and reduced albumin in serum. (A) percent of oxidized reduced albumin in healthy subjects (see Fig. 1). (B) theoretical percent levels of oxidized/reduced albumin determined considering both concentration and rate constant (i.e. the "*oxidizing/reducing power"*) of all low mass natural thiols and disulfides (Fig. 3). (C) theoretical

percent levels of oxidized/reduced albumin determined only considering the concentrations of all low mass natural thiols and disulfides (Fig. 3).

Fig. 5. Accessible surface area and B-factors of all cysteine residues of HSA. (A) total accessible surface area of cysteines in HSA. (B) B-factors of sulfur atoms of cysteines in HSA (see Materials and Methods). The red dotted line indicates the average value.

Fig. 6. The *in vivo* redox interplay between albumin and cysteine. Values of kinetic constants for Cys_{34} are reported for purified HSA (black) and human plasma (red) (see also Tables 1 and 2). The kinetic constant for the reaction between HSA-Cys_{34}-SOH and cysteine is taken from Ref. 20.