Hemoglobin glutathionylation can occur through cysteine sulfenic acid intermediate: Electrospray ionization LTQ-Orbitrap hybrid mass spectrometry studies☆

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ABSTRACT

Glutathionylated hemoglobin (Hb-SSG) is now recognized as a promising biomarker of systemic oxidative stress. Aim of this study is to gain a mechanistic insight into its formation. The ability of GSSG to form Hb-SSG through a thiol-disulfide exchange mechanism was firstly examined. For this purpose, GSSG (ranging from 0.23 to 230 μmol/g Hb, 15 μM–15 mM final concentrations) was incubated with 1 mM Hb and the relative content of Hb-SSG determined by direct infusion mass spectrometry (Orbitrap as analyzer). No detectable Hb-SSG was observed at a GSSG concentration range found in physiopathological conditions (0.13–0.23 μmol/g Hb). To reach a detectable Hb-SSG signal, the GSSG concentration was raised to 2.3 μmol/g Hb (0.5% relative abundance). The relative content of Hb-GSSG dose-dependently increased to 6% and 11% at 77 and 153 μmol/g Hb, respectively. The second step was to demonstrate whether Hb-SSG is formed through a sulfenic acid intermediate, a well-recognized mechanism of S-protein glutathionylation. Cys β93 sulfenic acid was found to be formed by oxidizing Hb with 1 mM H_2O_2, as demonstrated by direct infusion and LC–ESI-MS/MS experiments and using dimedone as derivatazing agent. When H_2O_2-treated Hb was incubated with physiological concentrations of GSH (9 μmol/g Hb), the corresponding Hb-SSG form was detected, reaching 15% of relative abundance. In summary, we here demonstrate that Hb glutathionylation can occur through a Cys sulfenic acid intermediate which is formed in oxidizing conditions. Hb glutathionylation is also mediated by a thiol-disulfide transfer mechanism, but this requires a concentration of GSSG which is far to be achieved in physiopathological conditions.

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1. Introduction

S-glutathionylation is a reversible post-transational modification with critical roles in sulfhydryl homeostasis and signal transduction. Protein glutathionylation increases globally during overt oxidative stress but selectively in the presence of reactive oxygen species (ROS) that are generated during physiological signaling [1]. Several proteins are targets of S-glutathionylation, including enzymes, transcription factors, oncogenes as well as transport proteins such as hemoglobin. Protein S-glutathionylation can occur by several reactions as recently reviewed by Dalle-Donne et al.

[2]. These include: (i) direct interaction between partially oxidized (activated) protein sulfhydryls, i.e., thiyl radical, sulfenic acid, or protein S-nitrosothiol (S-nitrosated protein) and GSH; (ii) thiol/disulfide exchange reactions between protein thiols and GSSG or glutathionylated proteins; (iii) reaction between protein thiols and intermediate S-nitrosothiols such as S-nitrosoglutathione; (iv) direct interaction between a free protein cysteinyl residue and GSH triggered by many oxidants.

Protein glutathionylation, besides to be studied for its role in signal transduction and cell homeostasis, is also receiving great attention as a potential marker of mild oxidative damage. Among others, Hb-SSG is now recognized as a promising bio-marker of systemic oxidative stress [3]. Significant increase of Hb-SSG content, in respect to its low value in healthy individuals, has been found in several oxidative stress-based diseases such as diabetes, hyperlipidemia, Friedreich ataxia and chronic renal failure [4–8]. The mechanism by which glutathione can react with Hb in in vivo conditions is still unclear. It has been suggested that Hb-SSG increases in diabetic patients by a disulfide exchange mechanism

between Cys β93 and GSSG [9]. However, protein glutathionylation via a thiol-disulfide exchange mechanism is not considered a likely mechanism in vivo because the intracellular ratio GSH:GSSG would have to decline dramatically (i.e. from 100:1 to 1:1) to achieve 50% conversion of protein-SH to protein-SSG [10].

Protein glutathionylation can also occurs via Cys sulfenic acid intermediates (Cys-SOH). This has been shown to occur, for instance, with protein phosphatase-1B [11]. This mechanism is now receiving a great attention by considering that protein sulfenic acids are detected in physiological conditions and increase in response to ROS [12]. Cys-SOH is a reactive intermediate and undergoes rapid condensation either with another protein thiol (as an intramolecular or intermolecular interaction) or with a low molecular mass thiol, like glutathione or cysteine, to form a disulfide bond, or with another sulfenic acid to form a thiosulfinate [RS(O)SR] [13]. Studies on mammalian protein tyrosine phosphatases and albumin support the likelihood of rapid conversion of protein sulfenic acid derivative to protein glutathionylated adduct by GSH [11,14]. Because RBC are characterized by a high concentration of GSH (9 μmol/g Hb by using artifact-free measurements) [15], and are continuously exposed to a flux of oxidants (i.e. H_2O_2) [16] able to react with accessible thiol to form the corresponding sulfenic acid derivative, it is plausible that Hb-SSG is formed through a sulfenic acid intermediate that rapidly reacts with GSH. However this mechanism is not currently supported since no direct evidence on the formation of Cys β93 sulfenic acid exists. Based on these premises, in order to gain a mechanistic insight into the hemoglobin glutathionylation, aim of the present study is to give a MS evidence that Cys β93 sulfenic acid is formed in H_2O_2-driven oxidative conditions, and that the corresponding glutathionylated form is generated in the presence of physiological concentrations of GSH. Moreover, the effect of GSSG on Hb glutathionylation will be also considered.

2. Materials and methods

2.1. Chemicals and reagents

Human hemoglobin [associated genes: HBA1(3039), HBA2(30-40), HBB(3043), HBD(3045), HBE1(3046), HBG1(3047), HBG2(30-48), HBQ1(3049), HBZ(3050)] was purchased from Sigma–Aldrich (Milan, Italy). Since this preparation was predominantly methemoglobin, before usage, Hb dissolved in 2 mM Tris–HCl (pH 7.5) was reduced by 1,4-dithioerythritol (DTT, 0.5 mM) and then dialyzed overnight with Milli-Q water. Sequence grade modified trypsin was obtained from Promega (Milan, Italy). LC–MS grade solvents (Chromasolv®) were purchased from Sigma–Aldrich (Milan, Italy). LC-grade water (18 mΩ) was prepared with a Milli-Q water purifica-

tion system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

2.2. Samples preparation

The effect of GSSG on Hb glutathionylation was studied by incubating 1 mM Hb solution with GSSG (0.23–230 μmol/g Hb, corresponding to a final concentration range of 15 μM–15 mM) in 50 mM NH_4HCO_3 buffer (pH 7.4) for 60 min at 37 °C. Oxidation of Hb was carried out by incubating 1 mM Hb in the presence of 1 mM of H_2O_2 at 4 °C for 30 min in 50 mM NH_4HCO_3 buffer (pH 7.4). In another set of experiments, H_2O_2-treated Hb or native Hb was incubated with GSH (9 μmol/g Hb) or 10 mM dimedone. After 60 min at 37 °C, 5 μl aliquots were analyzed by MS (direct infusion) or digested by trypsin as below detailed.

2.3. Direct infusion mass spectral analysis

To detect changes in protein mass of the β-globin subunit, samples prepared as above reported were analyzed by direct infusion on a LTQ-Orbitrap Hybrid Mass Spectrometer (Thermo Scientific, Milan, Italy) equipped with an Electrospray Finnigan Ion Max source. After incubation, samples (5 μl) were mixed with 1 ml of $H_2O:CH_3CN:HCOOH$ (50:50:0.1, v/v/v) and infused into the mass spectrometer at a flow rate of 3 μl/min. Each sample was analyzed in SIM mode using a m/z 1130–1160 mass range, under the following instrumental conditions: AGC target 5×10^4, 500 ms maximum inject time, 1 microscan, scan time 1.9 s, resolving power 100,000, positive ion mode, capillary temperature 270 °C; spray voltage applied to the needle 3.5 kV, capillary voltage 37 V, nebulizer gas (nitrogen) flow rate set at 5 (a.u.), acquisition time 1 min. Direct infusion ESI-MS spectra were deconvoluted by using Xtract for Qual Browser v. 2.0.7 (ThermoQuest, Milan, Italy).

2.4. NanoLC–MS/MS analysis

Tryptic peptide maps were generated by nanoLC–ESI-MS/MS analysis in data-dependent scan mode and dynamic exclusion in positive-ion mode. For sample preparation, 5 μl of 1 mM Hb solution prepared as above described, were diluted to 50 μl with 50 mM NH_4HCO_3 (pH 7.8), and digested with trypsin at a protease:protein ratio of 1:20 (w/w) at 37 °C overnight. To stop the protease activity, samples were spiked with trichloroacetic acid (10% final concentration), centrifuged at 18,000 × g for 10 min and aliquots of 2 μl of digested peptide mixtures were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray tandem mass spectrometry (ESI-MS/MS).
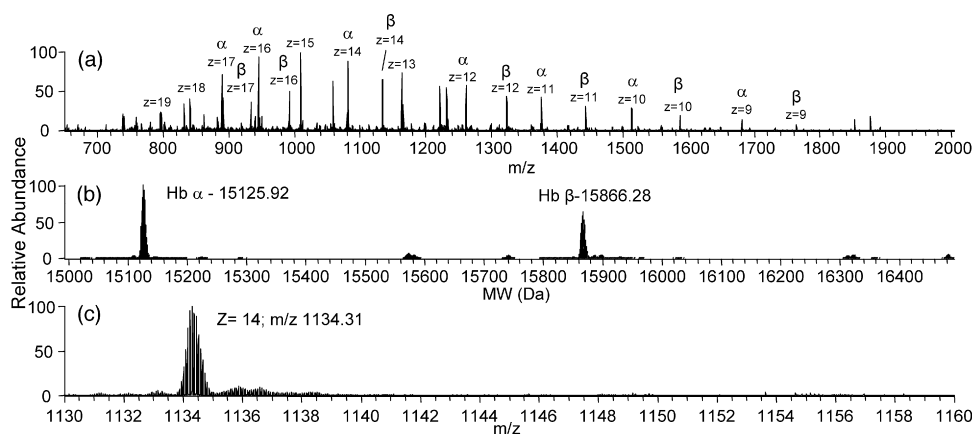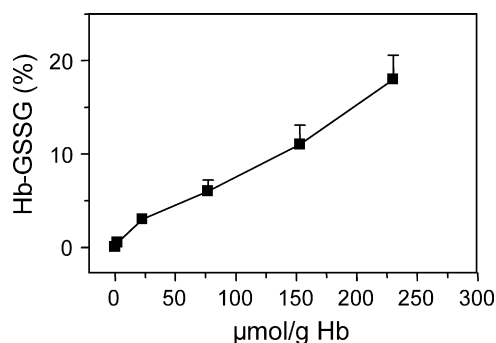

Fig. 1. Direct infusion ESI-MS analysis of human Hb. (Panel a) MS spectrum of Hb recorded in positive ion mode at a resolution of 100,000 (Orbitrap as analyzer). (Panel b) Deconvoluted spectrum; (Panel c) MS spectrum of Hb setting a m/z 1130–1160 scan range. The multicharged ion at m/z 1134.31 is relative to $z = 14$ of β globin.
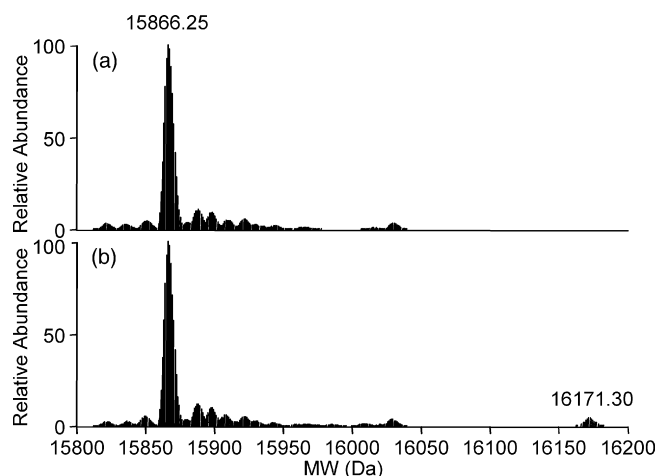
Fig. 2. GSSG induced Hb glutathionylation. Deconvoluted MS spectra of Hb (1 mM) incubated for 60 min at 37 °C in the presence of 0.23 μmol/g Hb (Panel a) and 2.3 μmol/g Hb (Panel b). Hb-SSG is detectable only in Panel b at 16171.3 Da.

Chromatography was performed using a Surveyor LC system (ThermoFinnigan, Milan, Italy) on a 180 μm × 10 cm column packed with 5 μm, Biobasic-18 stationary phase (Thermo, Superchrom, Milan, Italy). The pump flow rate was split 1:75 for a column flow rate of 2 μL/min. The column effluent was directly electrosprayed using the silica emitter source without further splitting. Mobile phase consisted of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The separation of peptides obtained by enzymatic digestion was achieved with a gradient elution from 0 to 60% B over 60 min. Before the next analysis, both the precolumn and the column were first washed with 90% solvent B for 10 min and then equilibrated with 100% solvent A for 20 min. For the identification of peptides, an LTQ XL-Orbitrap mass spectrometer was used (Thermo Scientific, Milan, Italy) and the electrospray interface (dynamic nanospray probe, Thermo Scientific, Milan, Italy) was set as follows: spray voltage 1.6 kV; capillary temperature 220 °C, capillary voltage 30 V; tube lens offset 120 V, no sheath or auxiliary gas flow. During analysis, the mass spectrometer continuously performed scan cycles in which first a high resolution (resolution r = 60,000 at m/z 400) full scan (250–2000 m/z) in profile mode was made by the Orbitrap, after which MS2 spectra were recorded in centroid mode for the three most intense ions (isolation width, 3 m/z; normalized collision energy, 30 CID arbitrary units). Protonated phthalates [dibutylphthlate (plasticizer), m/z 279.159086; bis(2-ethylhexyl)phthalate, m/z 391.284286] and polydimethylcyclosiloxane ions [$(Si(CH_3)_2O)_6+H]^+$; m/z 445.120025] were used for real time internal mass calibration [17]. Dynamic exclusion was enabled (repeat count, 3; repeat duration, 10 s; exclusion list size, 25; exclusion duration, 120 s; relative exclusion mass width, 5 ppm). Charge state screening and monoisotopic precursor selection was enabled, singly and unassigned charged ions were not fragmented. Peptide sequences were identified using the software turboSEQUEST (Bioworks 3.1, ThermoQuest, Milan, Italy), and using a database containing only human β and α globin. Xcalibur™ software provided instrument control and data analysis for the Thermo Electron mass spectrometer.

3. Results

Fig. 1a shows the ESI-MS spectrum of human hemoglobin recorded at a resolution of 100,000, characterized by several multicharged ions relative to the β and α chain (see the deconvoluted spectrum in Fig. 1b). The covalent modifications of the β chain were then studied by analyzing the z = 14 multicharged ions by setting a m/z 1130–1160 scan range (Fig. 1c), and then determining the MW of the adducts by an isotopic pattern deconvolution.

The first part of the work was to understand the role of GSSG on Hb glutathionylation (thiol-disulfide exchange mechanism), previously proposed to explain the high levels of Hb-SSG in diabetic patients [9]. Fig. 2a shows the deconvoluted MS spectra of the unmodified β chain of Hb (1 mM), characterized by a base peak at 15866.25. When Hb was incubated in presence of 23 μmol GSSG/g
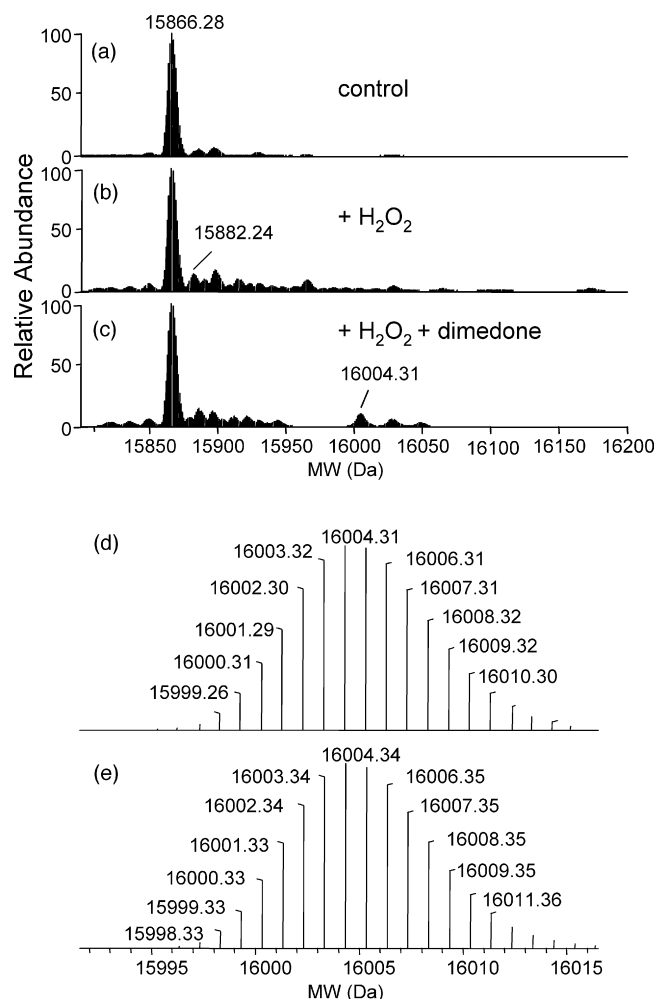
Fig. 4. H₂O₂ induces sulfenic acid formation in the β globin chain. (Panels a and b) Deconvoluted MS spectra of Hb incubated in the absence (a) and presence (b) of H₂O₂. (Panel c) Deconvoluted MS spectrum of Hb oxidized by H₂O₂ and then incubated for 60 min with dimedone at 37 °C. The dimedone adduct is detectable at 16004.31 Da. (Panels d and e) Experimental (d) and simulated (e) isotopic pattern distribution of the dimedone adduct at 16004.31 Da (mass accuracy 1.8 ppm).

**Fig. 3.** Dose-dependent effect of GSSG on Hb-SSG formation.
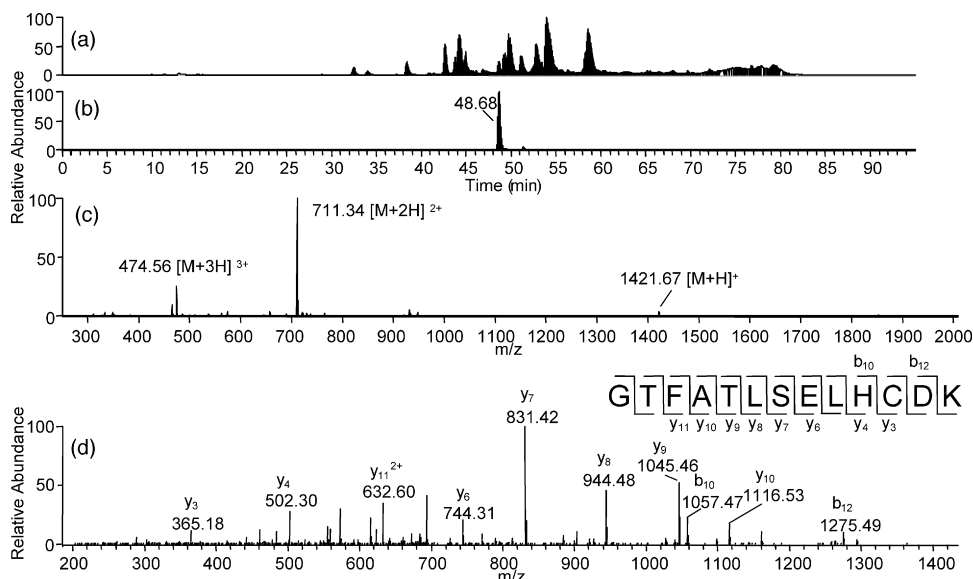
Fig. 5. LC–ESI-MS/MS analysis of native Hb. (Panel a) TIC of Hb digested in not reducing condition by trypsin; (Panel b) SIC obtained by setting the ion at m/z 711.34 as filter ion, the Cys β93 containing peptide (GTFATLSELHCDK) elutes at RT = 46.68; (Panel c) MS spectrum of the peptide GTFATLSELHCDK; (Panel d): MS/MS spectrum of the peptide GTFATLSELHCDK (parent ion at m/z 711.34).

Hb for 60 min at 37 °C (Fig. 2b), the Hb-SSG adduct was well detected at 16171.30 Da, reaching almost 3% of relative abundance (native β globin as base peak). The relative abundance of Hb-SSG was then studied in respect to different concentrations of GSSG (ranging from 0.23 to 230 μmol/g Hb), and taking as constant the final amount of Hb. No detectable adduct was observed when GSSG concentration was reduced to 0.23 μmol/g Hb, a concentration that was reported in some oxidative stress-based pathological conditions [18].

To reach a detectable Hb-SSG signal (0.5%), the GSSG concentration was raised to 2.3 μmol/g Hb. The relative content of Hb-SSG dose-dependently increased to 6% and 11% at 77 and 153 μmol/g Hb, respectively (Fig. 3).

The second part of the study was aimed to understand whether Hb glutathionylation can occur via a sulfenic acid intermediate, a well recognized mechanism of protein glutathionylation as reviewed by Mieyal et al. [10] and Dalle-Donne et al. [2]. To do this, we firstly studied the formation of Cys sulfenic acid intermediate by treating human Hb with H_2O_2, which resulted in a rapid and time-dependent oxidation of oxyHb to metHb, as spectrophotometrically determined according to the method of Van der Berg [19] (data not shown). As shown in Fig. 4b, H_2O_2 incubation induced several covalent modifications, and among these, that at 15882.24 Da, characterized by an increase of 16 Da in respect to native β globin (15866.25 Da, Fig. 4a). The β globin is characterized by several oxidizable residues, such as 2 Cys and one Met residue. To understand whether the oxygen addition is due to sulfenic acid, the reaction mixture was incubated with the nucleophilic reagent dimedone (5,5-dimethyl-1,3-cyclohexanedione), which reacts specifically with sulfenic acid to form a stable thioether adduct [20]. As shown in Fig. 4c, the deconvoluted
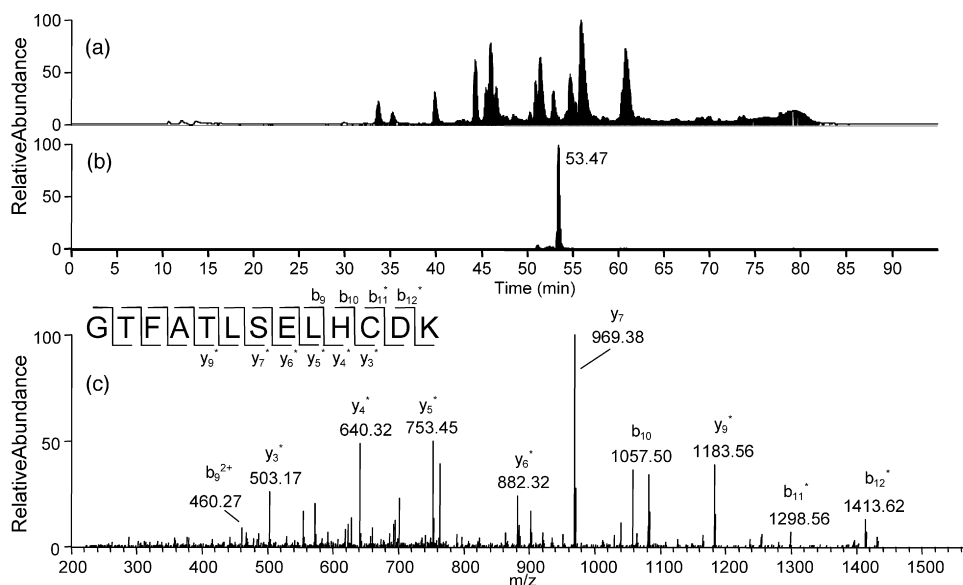
Fig. 6. LC–ESI-MS/MS analysis of Hb adducted by dimedone. (Panel a) TIC of trypsin digested Hb oxidized by H_2O_2 and then incubated with dimedone for 60 min at 37 °C; (Panel b) SIC obtained by setting the ion at m/z 780.37 as filter ion, the dimedone adducted peptide elutes at RT = 53.47; (Panel c) MS/MS spectrum of the dimedone adducted peptide (parent ion at m/z 780.37). The y and b fragments indicate that Cys β93 is the adduction site.

MS spectrum was characterized by an easily detectable peak at 16004.31 Da, due the addition of dimedone which, by contrast, was absent when native Hb was incubated in the presence of the derivatizing agent (data not shown). As a confirmation of the dimedone adduct, Fig. 4 shows the overlapping of the experimental (Fig. 4d) and simulated (Fig. 4e) isotopic pattern distribution. The results well demonstrate that H_2O_2 oxidation induces sulfenic acid formation in the β globin chain.

LC–ESI-MS/MS analysis of digested peptides demonstrates that the sulfenic acid occurs at the Cys β93 residue. In particular, Fig. 5a shows the TIC trace of digested native hemoglobin (sequence coverage of α and β globin: 92 and 96%, respectively). Peptide search by Sequest identified the trypsin peptide containing the Cys β93 (GTFATLSELHCDK) at RT = 48.7 min, characterized by a MS spectrum showing a base peak at m/z 711.34 $[M+2H]^{2+}$ (Fig. 5c). Fig. 5b shows the single ion chomatogram (SIC) obtained by plotting the current ion of the selected ion at m/z 711.34 (filter ion) vs. time. The peptide sequence was then confirmed by MS/MS analysis (Fig. 5d).

The TIC trace of digested hemoglobin oxidized by H_2O_2 and then incubated with dimedone is shown in Fig. 6a. The native peptide containing Cys β93 is significantly reduced (data not shown). By setting the dimedone adduct as a variable modification of Cys, the dimedone adduct of GTFATLSELHCDK was identified at RT = 53.47 min (the MS spectrum is characterized by a base peak at m/z 780.37, $z = 2$). Fig. 6b shows the SIC trace obtained by setting the ion at m/z 780.37 as filter ion. The attribution was confirmed by MS/MS analysis (Fig. 6c) and on the basis of the y and b fragments it was found that the sulfenic acid modification occurs at the Cys β93 residue. According to the nomenclature by Biemann, y and b fragments represents the C- and N-terminus fragment ions arising from the amide bond cleavage [21].

We then studied the role of sulfenic acid on the mechanism of Hb glutathionylation. When Hb was oxidized by 1 mM H_2O_2 and then treated with physiological concentrations of GSH (9 μmol/g Hb), Hb-SSG was detected at 16171.30 Da, reaching a relative abundance of 15% (Fig. 7a). No Hb-SSG adduct was observed when Hb was treated with H_2O_2, dimedone and then GSH (Fig. 7b), nor when Hb was incubated in the presence of GSH (Fig. 7c). As a confirmation of Hb-SSG, Fig. 7 shows the overlapping of experimental (Fig. 7d) and simulated (Fig. 7e) isotopic pattern distribution. These results well indicate that the sulfenic acid derivative of Cys β93 rapidly reacts with GSH to form the corresponding glutathionylated form.

Glutathionylation of Cys β93 was finally confirmed by LC–ESI-MS/MS analysis as shown in Fig. 8. In particular, setting S-glutathionylation as variable modification on Cys, the glutathionylated peptide GTFATLSELHCDK characterized by multicharged ions at m/z 576.25 ($z = 3$) and m/z 863.87 ($z = 2$) (RT = 53.47 min) was identified (Fig. 8a, TIC trace; Fig. 8b, SIC trace at m/z 863.87). MS/MS analysis (m/z 863.87 as parent ion) identified Cys β93 as the glutathionylated residue (Fig. 8c), as expected.

4. Discussion

The data here reported well demonstrate that Hb glutathionylation can occur through the Cys β93 sulfenic acid intermediate which is formed in oxidizing conditions. Hb glutathionylation also occurs by a thiol-disulfide transfer mechanism with GSSG as already known, but this requires a high concentration of the disulfide, a condition which is difficult to achieve in physiopathological conditions, and that should be verified by using validated assays.

Previous studies reported that the relative content of Hb-SSG determined by mass spectrometry ranges from 1.21 ± 0.12% [11] to 3.15 ± 1.99% [7] in control subjects, and significantly increases in diabetic (2.26 ± 0.29%), hemodialysis (9.99 ± 2.97%), transplant (4.69 ± 3.19%) and peritoneal dialysis (7.63 ± 1.81%) patients [7,9]. Our MS findings indicate that to reach both physiological and
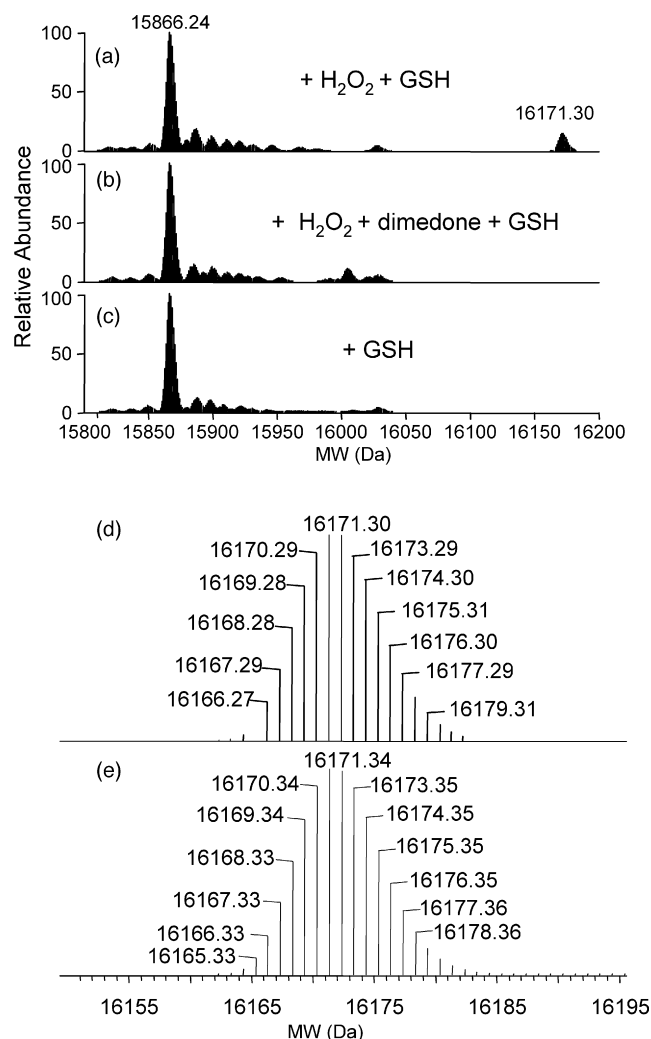
Fig. 7. Hb glutathionylation occurs through Cys β93 sulfenic acid intermediate. Deconvoluted MS spectra of Hb oxidized by 1 mM H_2O_2 and then incubated in the absence (Panel a) and presence (Panel b) of dimedone and then in the presence of GSH for 60 min at 37 °C. (Panel c) MS spectrum of Hb incubated in presence of GSH. (Panels d and e) Experimental and simulated isotopic pattern distribution of Hb-SSG (mass accuracy 2.4 ppm).

pathological Hb-SSG concentrations, a high content of GSSG is required, hardly achievable *in vivo*. In particular, the GSSG content in control subjects has been reported to be 0.13 μmol/g Hb and to significantly increase in some pathological conditions, such as peritoneal dialysis patients (0.23 μmol/g Hb) [18]. However these concentrations are still much lower than those able to induce Hb glutathionylation (≥2.3 μmol/g Hb), as shown in the present study. Moreover it should be considered that most of the GSSG values so far reported are overestimated since sample pretreatment with N-ethylmaleimide (NEM) required to prevent artifacts is usually omitted. In particular, by using a validated assay, where methodological artifacts were prevented with NEM, it has been reported a GSSG blood concentration of 10 nmol/g Hb in control subjects [15,22]. The results here reported agree with the considerations recently reported by Dalle-Donne et al. [23], suggesting that, although GSSG is capable of glutathionylation a number of proteins in vitro, thiol exchange is an unlikely intracellular mechanism for S-glutathionylation, because the GSSG levels measured in oxidant-treated cells are insufficient to trigger S-glutathionylation through a thiol/disulfide exchange reaction. We well understand that our experiments were carried out in a simple model and that several
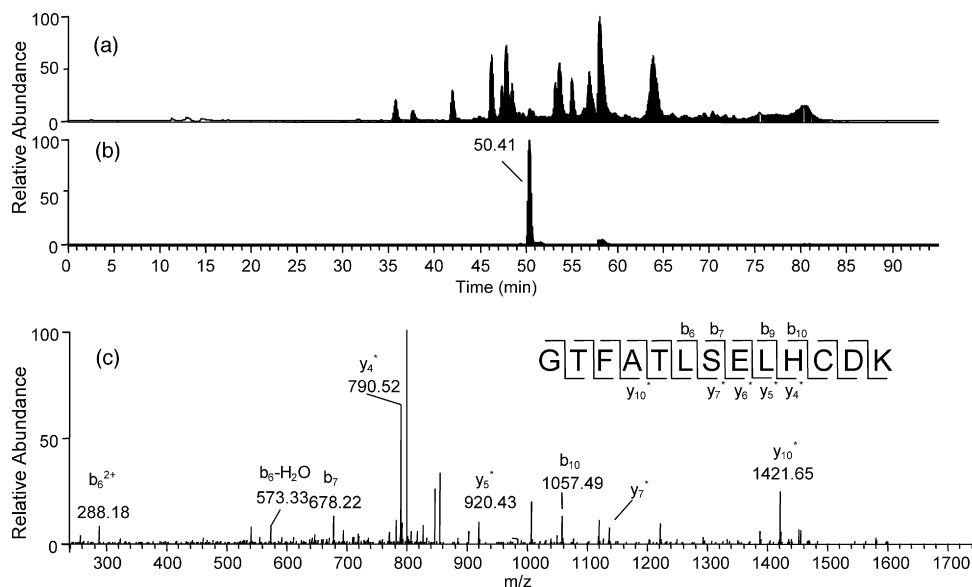
Fig. 8. LC–ESI-MS/MS analysis of Hb-SSG. (Panel a) TIC of trypsin digested Hb oxidized by H_2O_2 and then incubated with GSH for 60 min at 37 °C; (Panel b) SIC trace obtained by setting the ion at m/z 863.87 as filter ion, the GSH adducted peptide elutes at RT = 50.41 min; (Panel c) MS/MS spectrum of the GSH adducted peptide (parent ion at m/z 863.87). The y and b fragments indicate that Cys β93 is the adduction site.

factors could induce the thiol exchange in the RBC microenvironment. Further studies need to be carried out in intact RBCs in order to investigate the effective role of GSSG in Hb glutathionylation.

The second part of the study demonstrated that Hb glutathionylation can occur via sulfenic acid intermediate. In particular, we gave evidences, by using a MS approach, that oxidative conditions driven by H_2O_2 induce sulfenic acid formation of Cys93, which then reacts with physiological concentrations of GSH, leading to the formation of the corresponding GSH adduct. In the present study H_2O_2 was selected as oxidizing agent to generate sulfenic acid, but it should be underlined that other oxidizing agents which are known to be formed in RBCs, are able to form sulfenic acid, among which peroxynitrite [24]. Moreover S-nitroso (RSNO) Hb has been reported to act as sulfenic acid precursor [13]. Hence, Hb sulfenic acid can be potentially generated in RBCs through several pathways, but more studies are required to give direct evidences of Hb sulfenic acid formation in different physiopathological conditions.

Finally, we should underline that in the present work we focused our attention on the chemical modifications of Cys β93; however, preliminary MS results well indicate that also Cysβ112 and Cysα104 are oxidized to the corresponding sulfenic acid derivatives and that they react with GSH to form the corresponding glutathionylated form. MS and molecular modeling studies are currently addressed to confirm these preliminary results and to evaluate the order of reactivity.

Acknowledgements

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