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Formation of protein-derived electrophiles in ribonuclease A by biologically relevant oxidants

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ABSTRACT

Oxidative modifications in proteins have been extensively studied and found to increase in diabetes, cardiovascular diseases, neurodegenerative diseases, and aging. Some of the most studied modifications include the nitration of tyrosine and the formation of carbonyls in proteins. Tyrosine can also be oxidized to 3-(1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine (HOCHDA) by several biologically relevant systems, a product that is electrophilic and reactive to biological nucleophiles such as glutathione. Herein, we characterized the reaction of a peptide containing HOCHDA with fluorescein-tagged glutathione by HPLC and mass spectrometry. To explore the possibility that the formation of oxidation-derived electrophiles occurs in proteins, we oxidized the tyrosine-rich, small protein, ribonuclease A, by different biologically relevant oxidizing systems and used fluorescein-tagged glutathione as the nucleophilic reagent. Oxidation of ribonuclease A with singlet oxygen, known to generate HOCHDA efficiently, generated an electrophile that reacted with fluorescein-tagged glutathione and was resistant to reduction by dithiothreitol. The amount of fluorescein-glutathione attached to the protein was quantified by gel filtration HPLC. Other oxidants such as peroxyl radical (from AAPH), ferryl (from hydrogen peroxide reaction with Fe(II):EDTA), and peroxynitrite, also generated a modified protein that reacted with fluorescein-glutathione. Analysis by LC-MS/MS indicated the formation of mono-oxygenated tyrosyl residues and di-oxygenated histidyl residues after exposure of the protein to AAPH which are good candidates to be the electrophilic centers. The formation of electrophiles was a common feature in the reactions of oxidants with ribonuclease A and may constitute an underappreciated mechanism of protein oxidative modification.

1. Introduction

As a result of aerobic metabolism, humans can produce a wide range of oxidants derived from oxygen and nitric oxide that can generate different chemical modifications in proteins [1–5]. The accumulation of these modifications leads eventually to the loss or even gain of protein function [2–4]. The damaged proteins are usually removed by the 20S proteasome and other proteases [4–6], but an increase in oxidized proteins is observed during aging and pathologies such as diabetes, cardiovascular and neurodegenerative diseases [2,3,5,6]. Furthermore, it has been observed that oxidized and covalently linked protein aggregates can inhibit normal protein degradation systems and thus

Abbreviations: HPOCHDA, 3-(1-hydroperoxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine; HOCHDA, 3-(1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine; HOHICA, (2S)-3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indole-2-carboxylic acid; LPOCHDA, peroxide-bridged tyrosine-lipid product (3-(1-lipoperoxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine; DAP, Diels-Alder cyclization tricyclic endproduct; Fl-GSH, Fluorescein-tagged glutathione; $(Fl\text{-}GS)_2$, Fluorescein-tagged glutathione disulfide; NHS-Fluorescein, N-hydroxysuccinimide-fluorescein; TFA, triluoroacetic acid; AUC, area under the curve; AAPH, 2,2′-Azobis(2-amidinopropane) dihydrochloride; GF-HPLC, gel filtration high performance liquid chromatography.

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constitute a vicious cycle leading to the accumulation of oxidized, unfolded, and aggregated proteins [4–6].

The oxidants produced in biological systems show different reactivities and selectivities to certain amino acid residues. Hydroxyl radical, produced by homolysis of peroxynitrite, some Fenton-like reactions, or by gamma radiolysis of water, is the least selective species because of its very high reactivity and reacts with most amino acids, leading to hydroxylation, peptide side chain fragmentation, and protein backbone fragmentation [2,4,7]. Hydrogen peroxide, on the other hand, reacts mainly with thiolates in cysteines, and also methionine but more slowly [8]. Peroxynitrite as such also reacts preferentially with thiolates, but its derived radical nitrogen dioxide also reacts with tryptophan and tyrosine [2,9]. Carbonate radical, produced by the homolysis of the peroxynitrite adduct with carbon dioxide, the reaction of hydroxyl radical with bicarbonate, and the peroxidase activity of some enzymes in the presence of bicarbonate, reacts preferentially with tryptophan, thiols and tyrosine [2,10]. The final products of such reactions include sulfenic acid, disulfides, sulfinic, and sulfonic acid derivatives of cysteine, crosslinked, hydroxylated, and nitrated tyrosine, to name a few [2,4,5,11].

Some modifications, such as 3-nitro-tyrosine are stable and have been used as evidence of the increase in protein oxidation in aging and in various disease states, including cardiovascular and neurodegenerative diseases [12–15]. The nitration of tyrosine occurs in two steps and involves the formation of tyrosyl radical and the subsequent addition of nitrogen dioxide to generate 3-nitro-tyrosine [2]. Other products of tyrosine are generated in parallel. The reaction of two tyrosyl radicals yields 3,3′-dityrosine, whereas the reaction of the tyrosyl radical with superoxide can lead to "tyrosine hydroperoxide" [16–18].

In contrast to the most common oxidation products of tyrosine, "tyrosine hydroperoxide" is not aromatic, and is formed by the preferential binding of oxygen to the C1 atom in the ring, and is identical to the product of tyrosine with singlet oxygen (Fig. 1) [19–21]. A more appropriate name is 3-(1-hydroperoxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine (HPOCHDA). This HPOCHDA can be reduced to yield the corresponding alcohol 3-(1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine (HOCHDA), which can cyclize by an intramolecular Michael addition to generate (2S)-3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indole-2-carboxylic acid (HOHICA, Fig. 1) [20]. Furthermore, the reaction of tyrosyl radical with lipid peroxyl radicals can generate a peroxide-bridged tyrosine-lipid product (3-(1-lipoperoxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine, LPOCHDA) that can undergo an intramolecular Diels-Alder cyclization to generate a tricyclic end product (DAP, Fig. 1) [11]. These alternative products of tyrosine oxidation have been less studied, though they are very likely to be formed in sites of inflammation. Besides, these products are reactive and could undergo further reactions that contribute to the cellular damage caused by oxidation.

These alternative products all have α,β-unsaturated carbonyls that are susceptible to Michael additions by nucleophiles such as thiols. Adducts of HOCHDA with glutathione have been evidenced in small peptides and also in proteins [22]. The reaction of a dipeptide containing HOCHDA with GSH was found to occur with k ~0.16 M^{-1} s^{-1}, and to be reversible (k_{off} ~ 2 × 10^{-5} s^{-1}) [22]. In proteins, the HPOCHDA was generated from the reaction of tyrosyl radical with superoxide, and shown to occur preferentially at Tyr151 in myoglobin and Tyr14 in insulin, and generated glutathione adducts that were identified by mass spectrometry [22,23].

The formation of electrophiles in proteins is very interesting because amino acid residues in proteins are most of the time not electrophilic, but rather neutral or nucleophilic. The occurrence of electrophiles in proteins is basically restricted to sulfenic acids, disulfides, and persulfides in cysteine residues [24–26]. In case of oxidation by the potent oxidant hydroxyl radical, protein side-group and backbone fragmentation can lead to the generation of aldehydes that can then react with amines from lysines to form Schiff bases and protein cross-linking. Alternatively, the formation of electrophiles from biological oxidative stress can derive from the oxidation of lipids, which can generate

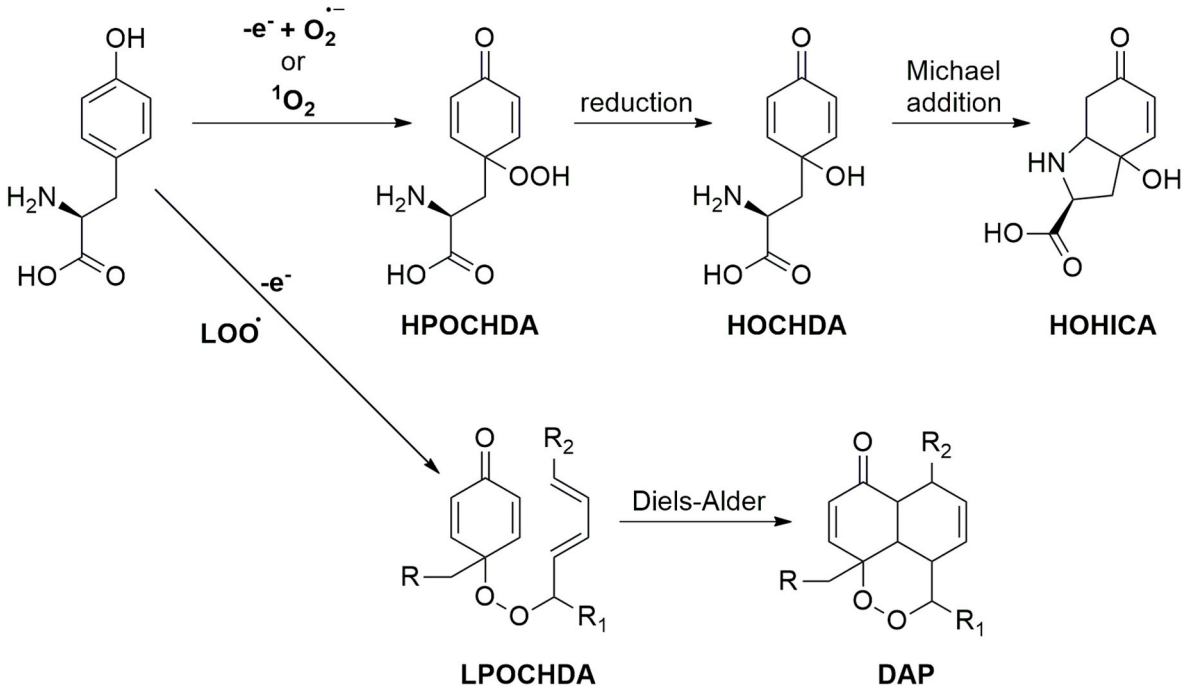


Fig. 1. Alternative products of tyrosine oxidation. Tyrosine reacts either with singlet oxygen or is oxidized to tyrosyl radical that then reacts with superoxide to generate 3-(1-hydroperoxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine (HPOCHDA). This HPOCHDA can be reduced to yield the corresponding alcohol 3-(1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine (HOCHDA), which can cyclize by an intramolecular Michael addition to generate (2S)-3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indole-2-carboxylic acid (HOHICA). The reaction of tyrosyl radical with polyunsaturated lipid-derived peroxyl radicals generates 3-(1-lipoperoxy-4-oxocyclohexa-2,5-dien-1-yl)-L-alanine (LPOCHDA) that may undergo an intramolecular Diels-Alder cyclization to generate the tricyclic product DAP.

hydroxynonenal, nitrolipids, and other derivatives that have been shown to trigger cellular responses such as the antioxidant response, by reacting with thiols in sensor protein Keap-1 and activating Nrf2 [27]. Therefore, the formation of electrophiles in proteins would be detrimental because of protein crosslinking and aggregation but could also trigger cellular responses via Nrf-2. This modification is thus potentially very relevant in physiology and pathology and warrants further studies.

Herein, we aimed to evaluate the formation of electrophiles in a protein as a result of oxidation by biologically relevant oxidants such as singlet oxygen, peroxyl radical, ferryl, and peroxynitrite. To do this, our method was based on the reaction of electrophiles with thiols. First, we studied the reaction between the peptide glycyl-HOCHDA (G-HOCHDA) and fluorescein-derivatized glutathione (Fl-GSH). Then we used the protein ribonuclease A because it is tyrosine-rich and has no tryptophan residues. We optimized the detection of Fl-GSH adducts with singlet oxygen-oxidized ribonuclease A and studied the formation of adducts in ribonuclease A exposed to different oxidants. Finally, sites of oxidation were identified by mass spectrometry.

2. Materials and methods

Materials. Reagents were obtained from Sigma, Applichem, Thermo, and GE Healthcare. Peroxynitrite was synthesized as described in Ref. [28]. Absorbance measurements were done in a Cary 50 UV–Vis spectrophotometer (Varian). HPLC analysis was done in an Agilent Infinity 1260, equipped with diode array UV–Vis detection (1260 DAD VL) and fluorescence detection (1260 FLD).

Synthesis of Fluorescein-Glutathione. Fluorescein-labeled glutathione (Fl-GSH) was prepared by treating 6 mM glutathione disulfide (GSSG) in 50 mM borate buffer at pH 8.5, with 3 mM N-hydroxysuccinimide-fluorescein (NHS–F, Pierce/Thermo, Rockford IL) prepared in dimethylformamide (DMF) for 1 h, at room temperature, protected from light. Fl-GSH was obtained by reduction with 40 mM DTT for 1 h, at room temperature and mechanical agitation. Fl-GSH was purified by solid-phase extraction using a Bakerbond C18, 500 mg cartridge. The sample was acidified with 1 % trifluoroacetic acid (TFA) and then applied to the cartridge. The cartridge was washed with increasing concentrations of acetonitrile in 0.1 % TFA water, and the elution samples were then analyzed by RP-HPLC. The fractions corresponding to Fl-GSH were then pooled and quantified by absorbance at 495 nm at pH 8.5 using $\varepsilon = 70000\ M^{-1}cm^{-1}$.

Reaction between oxidized peptide and Fl-GSH. The reaction was done by mixing 200 μM G-HOCHDA with 0.2, 0.6, 2.0, and 4.0 mM Fl-GSH in 100 mM sodium phosphate, 0.1 mM DTPA pH 7.4 for 2 h at room temperature, and protected from light. Analysis of the products was done by RP-HPLC, at 1 mL/min using the following gradient: (0–5)' 0 % B, (15-17)' 100 % B, (18-23)' 0 % B. Mobile phases were A: 0.1 % trifluoroacetic acid (TFA) in water and B: 0.1 % TFA in acetonitrile, using an Agilent Eclipse Plus C18, 100 × 4.6 mm column.

Mass spectrometric analysis of Fl-GSH and G-HOCHDA. Fractions collected from the HPLC underwent two cycles of evaporation in a vacuum concentrator to remove the TFA and were re-dissolved in ultrapure water. The aqueous samples were then diluted in 0.1 % formic acid and analyzed by direct infusion into a hybrid triple-quadrupole/linear ion trap mass spectrometer (QTRAP4500, ABSciex). Mass spectrometer parameters were optimized for best signal quality. For molecular ion identification, Q1 and enhanced MS modes were employed in positive mode, electrospray voltage and declustering potential were set to 5.5 kV and 70 V, respectively, and the scan speed was set to 1000 Da/s for Q1 mode, and 250 Da/s for enhanced MS mode. Fragmentation experiments were performed in the Product Ion mode with a collision energy ramp from 20 to 50 V. Data were acquired with Analyst 1.6.2 software (ABSciex) and analyzed with PeakView 2.2 (ABSciex).

Oxidation of ribonuclease A. Ribonuclease A was oxidized by different systems. Oxidation by singlet oxygen was done using 100 μM ribonuclease A with 30 μM methylene blue either in water or deuterated water, in an ice bath, flushing with pure oxygen, under illumination (two fluorescent lamps of 1950 lm) for 1 h.

Oxidation by peroxyl radical was done by reacting 100 μM ribonuclease A in 50 mM sodium phosphate, 0.1 mM DTPA, pH 7.4 at 37 °C with 10 and 100 mM AAPH for 1 h. The samples (0.5 mL) were in 15 mL tubes and stirred every 10 min to equilibrate with air and reoxygenate the solution. The AAPH was then removed from the solution using 5 kDa MWCO spin filters and at least 3 washes (GE Healthcare).

Oxidation with the Fenton-like system, that generates mainly the ferryl (Fe(IV)=O) EDTA complex, which hydroxylates tyrosine [29,30], was done using 100 μM ribonuclease A in 5 mM sodium phosphate pH 7.4, with either 0.2 mM H_2O_2 and 0.2 mM Fe(II):EDTA (1:2) or 4 mM H_2O_2 and 4 mM Fe(II):EDTA (1:2) as done previously with peptides [19]. The pH of EDTA was corrected to pH 7.4 so that the final pH was not affected. After 5 min, the protein was separated from the iron and the remaining H_2O_2 using 5 kDa MWCO spin filters (2 washing cycles).

Peroxynitrite oxidation was done using 100 μM ribonuclease A in either 50 mM sodium phosphate, 0.1 mM DTPA, pH 7.4 or 50 mM sodium phosphate, 50 mM sodium carbonate, 0.1 mM DTPA, pH 7.4, adding 1 mM peroxynitrite to a vigorously mixed protein solution. The peroxynitrite stock solution was pretreated with manganese dioxide to eliminate contaminating H_2O_2 and the concentration was determined by its absorbance at 302 nm ($\varepsilon = 1670\ M^{-1}cm^{-1}$) [31].

Reaction of Fl-GSH with oxidized protein. In all cases, 50 μM native or oxidized ribonuclease A was reacted with 2 mM Fl-GSH in 100 mM sodium phosphate, 0.1 mM DTPA, pH 7.4 for 2 h, protected from light and mixed by rocking at room temperature. To quantify the adducts, native ribonuclease A was labeled directly with NHS-fluorescein. The reaction was done in 100 mM sodium borate, pH 8.5, containing 200 μM ribonuclease A and 3 mM NHS-fluorescein for 1 h, at room temperature, protected from light. The labeled protein was then purified by gel filtration using a PD-10 desalting column (GE Healthcare) using 50 mM sodium phosphate, 150 mM NaCl pH 7.4 as the mobile phase. The concentration of fluorescein attached to the protein was quantified by absorbance at 495 nm ($\varepsilon = 70000\ M^{-1}cm^{-1}$) and then used as standards in HPLC.

Protein HPLC analysis. The formation of protein adducts with Fl-GSH in the different samples was studied by gel filtration in an HPLC (GF-HPLC) with UV–Vis and fluorescence detection. Before the injection, the sample was pretreated with 20 mM DTT for 30 min, to remove any possible mixed disulfides between the protein and Fl-GSH and to reduce the disulfide $(Fl\text{-}GS)_2$ that eluted closer to the protein peak. The different components were analyzed by following absorbance at 210, 254, 280, 450 and 495 nm, whereas fluorescence detection was done using $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm. The optimization of the method indicated that the best separation between Fl-GSH and ribonuclease A was achieved using two 5 mL HiTrap desalting columns (GE Healthcare) in series, at 25 °C. The mobile phase was 50 mM sodium phosphate, 150 mM NaCl pH 7.4, at a flow rate of 2.5 mL/min. The area under the curve (AUC) for absorbance at 495 nm was used to quantify protein-Fl-GSH adduct. The proportion of protein forming adduct with Fl-GSH ($F/Rnase_0$) was calculated by dividing the AUC observed for the Fl-GSH adduct over the AUC for fluorescein-labeled ribonuclease A (50 μM fluorescein).

Protein analysis by SDS-PAGE. The samples for electrophoresis (native or oxidized ribonuclease A reacted with Fl-GSH as described above) were treated with 10 mM sodium borohydride for 30 min at room temperature protected from light, to reduce protein carbonyls and prevent loss of Fl-GSH adducts, and then denatured by SDS (1 % w/v) and reduced with mercaptoethanol (2.5 % v/v) at 100 °C for 3 min. The SDS-PAGE was done in a 15 % polyacrylamide gel, and either stained using colloidal Coomassie blue or analyzed for fluorescence using a G:BOX F3 (Syngene).

Mass spectrometric analysis of native and oxidized ribonuclease A. Ribonuclease A was oxidized by peroxyl radical, as described above by incubating with 100 mM AAPH. The oxidation products were

determined in peptides generated by tryptic digestion, following the protocol of Leinisch et al., which uses a filter-aided sample preparation (FASP) method [32]. Briefly, oxidized protein and control samples were transferred in quadruplicates (150 μg protein) to spin filters (10 kDa cutoff), and phosphate buffer with or without AAPH was removed by centrifugation and replaced with 100 mM ammonium bicarbonate. This washing was repeated 3 times for each sample. Reduction and alkylation were performed by adding 100 μL of a solution of 100 mM ammonium bicarbonate, 10 mM TCEP, 40 mM chloroacetamide, and the samples were then incubated for 10 min at 95 °C with shaking. Subsequently, the alkylation solution was removed by centrifugation, 30 μL of 100 mM ammonium bicarbonate buffer, 0.1 mg/mL trypsin, 0.2 % deoxycholic acid were added, and the samples were incubated overnight at 37 °C with shaking. Next, 100 μL of Milli-Q water was added and tryptic peptides were collected by centrifugation. Deoxycholic acid was precipitated by adding 1 μL of formic acid solution (1 %; 265 mM), and then the samples were centrifuged and 50 μL of the supernatant was preserved for analysis. Next, the samples were desalted and concentrated using C18 StageTips, and then resuspended in 12 μL of mobile phase A (0.1 % formic acid in MS-quality water). The samples were analyzed on an Orbitrap Exploris™ 240 Mass spectrometer (Thermo) coupled with a Nano HPLC (Ultimate 3000, Thermo) with Thermo Scientific™EASY-Spray™ HPLC Columns Catalog number: ES900_C20442712. 5 μL of each sample was injected with technical replicate and eluted using a solvent gradient system over 60 min, starting with 95 % mobile phase A and 5 % (mobile phase B) 0.1 % formic acid in acetonitrile, up to 99 % Phase B at a flow rate of 0.2 μL/min.

Data were analyzed with PatternLab V (version 5.0.0.171) with High - High resolution and fully specific tryptic constraints, allowing for up to

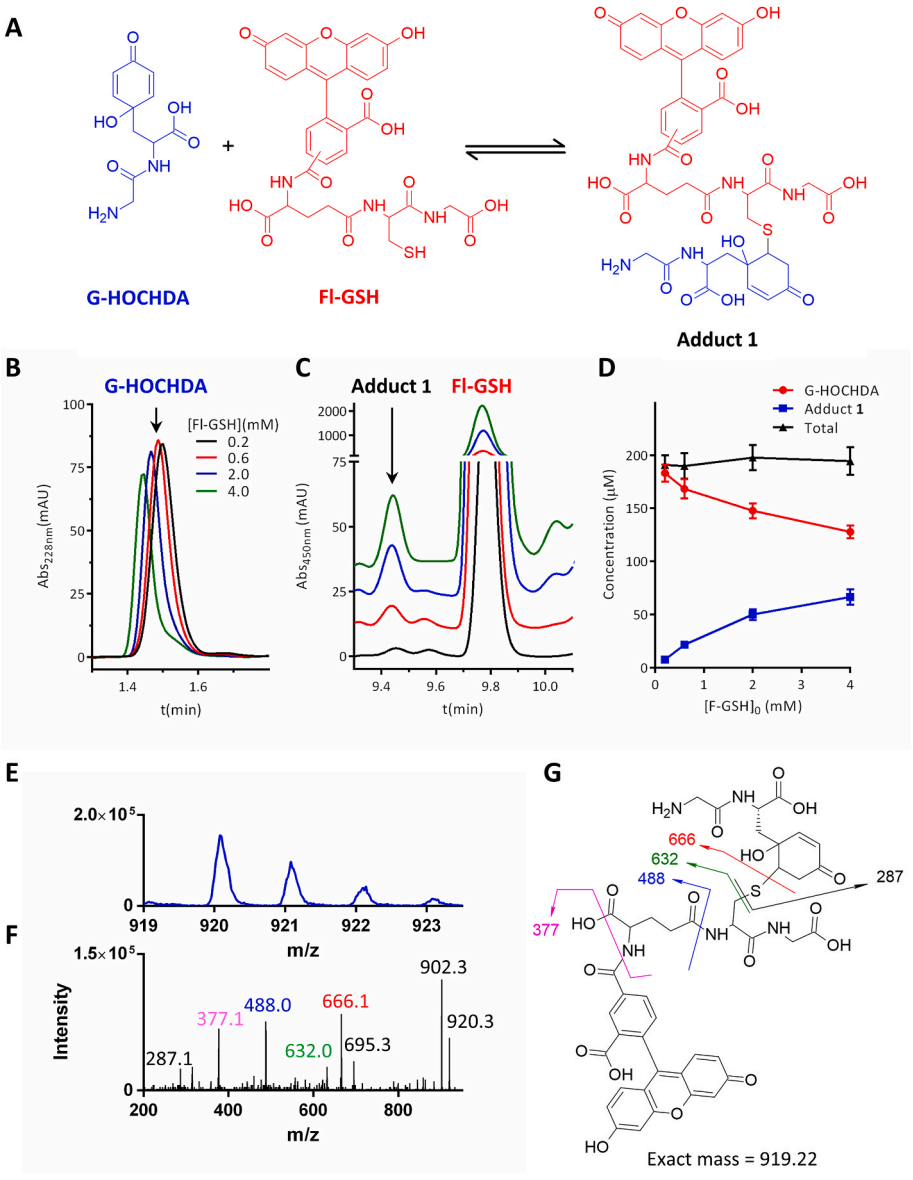


Fig. 2. Characterization of the reaction between G-HOCHDA and Fl-GSH. A) Reaction between G-HOCHDA and Fl-GSH to yield the Adduct **1. B) HPLC Analysis of the reaction between G-HOCHDA and Fl-GSH. G-HOCHDA (200 μM) and variable amounts of Fl-GSH reacted for 2 h and then the different species were separated by reversed-phase HPLC. G-HOCHDA was quantified by determining the area under the curve (AUC) at 228 nm of the peak eluting at 1.5 min (shifted in x-axis for clarity). C) Adduct 1 (9.45 min) and remaining Fl-GSH (9.8 min) concentration were determined by measuring the AUC at 450 nm (shifted in y-axis). D) Distribution of G-HOCHDA and adduct 1 after 2 h as a function of initial Fl-GSH concentration (representative result of 3 independent experiments). E)** Mass spectrometry characterization (enhanced MS1) of adduct **1. F)** Collision induced dissociation of adduct **1. G)** Structure of Adduct **1** and assigned fragmentations.

2 missed cleavages, and the Bos taurus UniProtKB database (version 2024_08_10). Cysteine carbamidomethylation was used as a fixed modification and oxidative modifications with changes in Met (+15.9949 Da), His (+15.9949 Da, +31.9898 Da), and Tyr (+15.9949 Da) were applied as variable modifications, with up to 3 modifications allowed per peptide. Differentially represented peptides in both conditions were identified using the TFold module of PatternLab with a Benjamini-Hochberg (FDR) q-value of 0.05, F-Stringency of 0.10, and L-Stringency of 0.40. Because we considered up to two missed cleavages, the same residue of interest appeared oxidized in different peptides. To compare the degree of oxidation of each Tyr and His residues, we calculated the ratio of the sum of spectral counts of peptides with one specific residue oxidized (AAx + mod) relative to the sum of all spectral counts of peptides corresponding to ribonuclease A: % AAox SC = [Σ(Spectral count of peptides with AAx + mod)/Σ(Spectral count of ribonuclease A peptides)] × 100.

3. Results

3.1. Reaction between G-HOCHDA and Fl-GSH

The oxidized dipeptide derived from glycyl-tyrosine, G-HOCHDA reacted with the fluorescent glutathione derivative Fl-GSH to form the Michael adduct **1**, as evidenced by HPLC (Fig. 2). The Michael adduct **1** is formed by the nucleophilic attack of the thiol on a β-carbon in one of the α,β-unsaturated bonds in the ring of the HOCHDA (Fig. 2A).

The chromatogram shows that the final concentration of G-HOCHDA decreases at higher initial concentrations of Fl-GSH (Fig. 2B, rt = 1.5 min), whereas there is an increase in the formation of the adduct **1** (Fig. 2C, rt = 9.4 min, and Fig. 2C). The reaction between G-HOCHDA and Fl-GSH did not proceed any further (yield of adduct **1** at 24 h did not increase), indicating that the reaction is reversible and that equilibrium concentrations are reached within 2 h. The estimated equilibrium constant is 5.9 mM, indicating that the formation of the adduct **1**, although occurring at measurable extents, is not thermodynamically favorable.

The formation of adduct **1** was confirmed by mass spectrometry (Fig. 2E–G). The peaks for *m/z* 920, 921 and 922 were observed at MS1, that corresponded to the single charge protonated adduct **1** and the expected isotopic distribution. The collision induced dissociation of the ion at *m/z* 920.1 yielded fragments with *m/z* 666, 632, 488 and 377, corresponding to the reverse of the Michael addition and protonated Fl-GSH (*m/z* = 666), to the loss of the sulfur atom from Fl-GSH (*m/z* = 632), and to the fragmentation of the peptide bond between the glutamate and the cysteine in Fl-GSH (*m/z* = 488). Other fragments included the G-HOCHDA plus sulfur (*m/z* = 287) and the carboxy-fluorescein resulting from the hydrolysis of the amide bond with the glutathione (*m/z* = 377).

Although the fluorescent probe Fl-GSH did not react completely but reached an equilibrium, it still worked to detect the electrophilic derivative in the small peptide GY with high sensitivity. Therefore, we proceeded to use this probe with a protein in an attempt to observe the formation of electrophilic sites as a result of oxidative modifications.

3.2. Formation of electrophiles in ribonuclease A

For further studies, the protein ribonuclease A was chosen. This protein is relatively small (13.7 kDa) and contains 6 tyrosine, 4 histidine and no tryptophan residues, all of which may form electrophilic products after oxidation. The formation of electrophilic groups in ribonuclease A exposed to different oxidants was studied by reaction with Fl-GSH and HPLC analysis.

3.2.1. Oxidation by singlet oxygen

In previous assays, singlet oxygen yielded the greatest amounts of HPOCHDA and HOCHDA from model peptides [19], thus, it was used for initial experiments and optimizing separation and detection of the oxidized proteins. The goal was to obtain all the protein in a single peak, separated from Fl-GSH. The best separation of ribonuclease A and Fl-GSH was achieved using two desalting Hi-Trap columns (GE) in series. Monitoring absorbance and fluorescence of both protein and fluorescein, it was observed that the protein eluted early at 1.4 min, whereas the free Fl-GSH eluted in a broad peak with retention times greater than 2.5 min. An advantage of this method is the short time needed for each analysis. Fig. 3 shows that the native protein did not form adducts with Fl-GSH, as no signal was observed by absorbance at 495 nm or fluorescence (EX 490, EM 520 nm) at 1.4 min (Fig. 3). Also, it could be seen that the oxidation of ribonuclease A by singlet oxygen in deuterated water led to a higher degree of adduction by Fl-GSH in comparison to the one exposed to singlet oxygen in regular water (peak at 1.4 min). This was expected because the half-life of singlet oxygen in deuterated water increases over ten times thus increasing its effective concentration and oxidation yield [33]. As indicated in the methods section, before GF-HPLC, the sample was treated with DTT for two reasons, to eliminate potential mixed disulfides between Fl-GSH and the protein that could give false positive signals and to reduce the disulfide $(Fl\text{-}GS)_2$ that interferes with the chromatogram baseline of the protein. DTT treatment may also have led to a small loss of labile F-GSH adducts, because of the reversibility of the reaction, but improved considerably the baseline (not shown). At this point we could not differentiate between mixed disulfides and labile adducts, so it is possible that our approach underestimated the amount of electrophiles formed.

The oxidation of ribonuclease A by singlet oxygen in D_2O and its labeling by Fl-GSH was also studied by SDS-PAGE, both by Coomassie staining and fluorescence (Fig. S1). To prevent the loss of the probe by the reverse reaction during sample preparation, $NaBH_4$ was used to reduce the carbonyl group to alcohol and lock the Fl-GSH to the oxidized

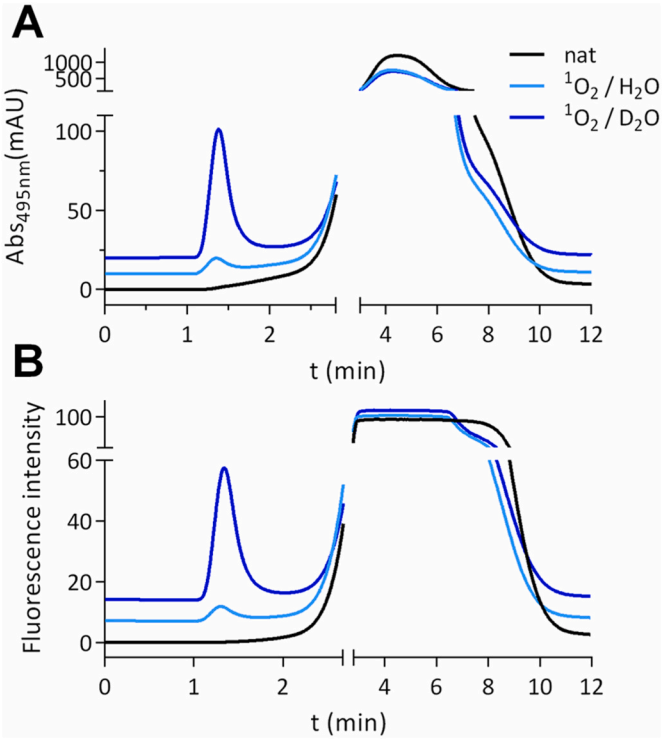


Fig. 3. Separation of ribonuclease A and Fl-GSH by gel filtration chromatography (GF-HPLC). A) Ribonuclease A in native form and oxidized by singlet oxygen in either regular or deuterated water were reacted with Fl-GSH and analyzed by gel filtration chromatography. The protein eluted at 1.4 min and the Fl-GSH at times greater than 2.5 min. The formation of adducts between Fl-GSH and ribonuclease A was evidenced by a signal at 495 nm at 1.4 min (signal shifted in y-axis). **B)** The sample was also analyzed by fluorescence (EX 490, EM 520 nm) and similar results were observed.

residue. The oxidation of ribonuclease A by singlet oxygen had an effect on electrophoretic mobility, evidencing significant damage to the protein and formation of covalent dimers. On the other hand, the addition of Fl-GSH had no effect on the electrophoretic mobility of either native or oxidized ribonuclease (Fig. S1A). In preliminary experiments, we observed that analyzing the proteins without additional treatment produced a significant fluorescence signal from the native protein, whereas standard treatment with 2-mercaptoethanol (3 min at 100 °C) led to a loss of fluorescence signal from both native and oxidized protein. Pretreating with $NaBH_4$ before 2-mercaptoethanol produced the greatest contrast in fluorescence signal between oxidized and native protein, which is consistent (though not exclusively) with the reduction of carbonyl-containing Michael adducts and stabilization of the adducts (Fig. S1B). Even though the formation of the adduct between fluorescent Fl-GSH and oxidized protein could be observed by fluorescence in SDS-PAGE, the native protein also exhibited a small signal that could not be lowered. Therefore, to quantify the extent of formation of electrophiles in ribonuclease A by different oxidizing systems, the HPLC method was preferred because it involved less manipulation of the samples, a very clean background was observed with native protein, and allowed direct quantification of the labeling from the area under the curve of the chromatogram.

3.2.2. Oxidation by peroxyl radicals

In order to assess whether free radical oxidations were also capable of generating electrophiles in the protein, different biologically relevant oxidizing systems were evaluated. First, to mimic lipid peroxyl radicals, the azo initiator AAPH was used. The rate of peroxyl radical formation by AAPH is mainly determined by the temperature, and in lesser degree by the solvent and pH [34]. In water at 37 °C, the half-life of AAPH is 175 h, thus the rate of peroxyl radicals formation is nearly constant for the first hours. These assays were done with 10 and 100 mM AAPH for 1 h, equivalent to exposing the protein to 49 and 490 μM peroxyl radicals, respectively [34]. The HPLC analysis of the protein oxidized by peroxyl radical showed the formation of electrophiles in a dose-dependent manner (Fig. 4). The formation of adduct increased two-fold and not ten times as expected, probably because the protein became over-oxidized. Part of the electrophiles detected in the protein are expected to be formed from the reaction between tyrosine residues and peroxyl radicals, yielding LPOCHDA-like products, with electrophilic properties (Fig. 1) [11].

3.2.3. Oxidation by peroxynitrite

Ribonuclease A was oxidized by peroxynitrite ($ONOO^-$) in the absence or presence of CO_2. Peroxynitrite decomposes by homolysis to yield approximately 30 % nitrogen dioxide and hydroxyl radical, that are known to oxidize proteins, and lead to tyrosine nitration and hydroxylation [35]. In the presence of CO_2, the yield of nitrogen dioxide increases to 35 %, with equal amounts of carbonate radical, that have been observed to increase nitration yields and prevent hydroxylation [9]. The chromatograms showed that oxidation of ribonuclease A by $ONOO^-$ also leads to the formation of protein electrophile products that increase by addition of CO_2 (Fig. 4).

3.2.4. Oxidation by Fenton-like system

Next, the protein was oxidized with a Fenton-like system consisting of Fe(II):EDTA and H_2O_2 in two different concentrations (0.2 and 4 mM), to generate different amounts of oxidants. This reaction generates mainly ferryl (Fe(IV)=O) EDTA complex, that hydroxylates tyrosine (and phenylalanine) [30]. In tyrosine-containing peptides we showed that this system generated both 3,4-dihydroxyphenylalanine and HOCHDA, similar to what was expected for hydroxyl radical [19]. In ribonuclease A, this Fenton reaction generated electrophiles that were evidenced by HPLC by formation of adducts with Fl-GSH, which increased with the concentration of oxidant (Fig. 4). The adduct peak overlapped with an adjacent peak that corresponds to the disulfide

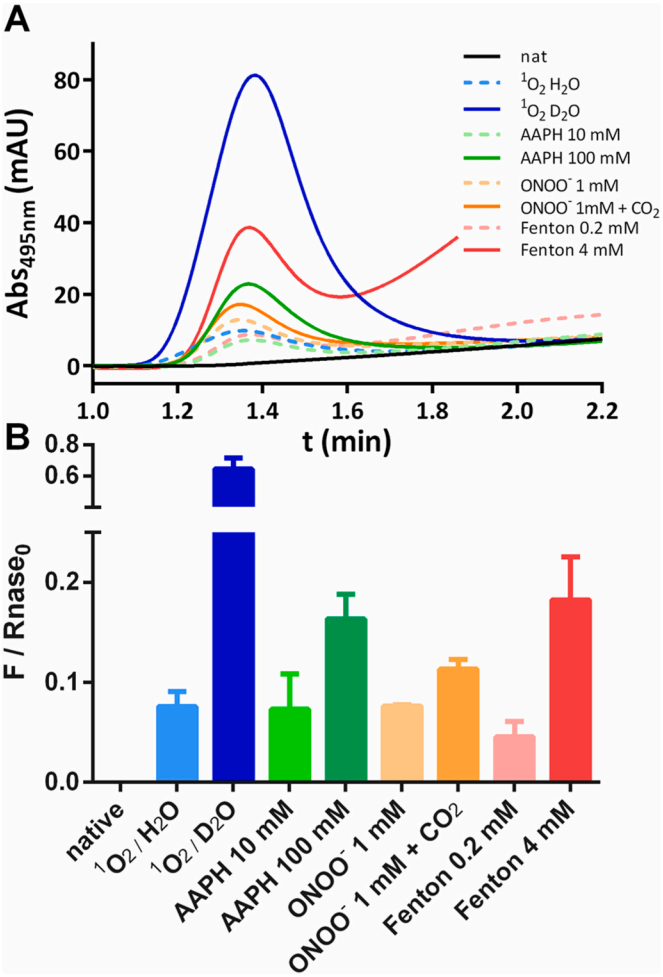


Fig. 4. Formation of adducts in ribonuclease A oxidized by different systems. A) The protein was separated from free Fl-GSH by gel filtration chromatography and elution was monitored following the absorbance at 495 nm, where only fluorescein absorbs. The figure shows the peak of the protein that elutes at 1.4 min and the different systems. Note that the native protein does not show a signal at 495 nm, indicating that it did not form adducts with Fl-GSH, whereas all the other oxidizing systems led to the formation of adducts. **B)** The amount of adduct formed per oxidizing system was quantified using a standardized ribonuclease A covalently labeled with NHS-fluorescein. The measurements were done by duplicate from three different experiments (n = 3 duplicates), and the results are expressed as the average plus minus the standard deviation.

$(Fl\text{-}GS)_2$, that could not be completely reduced by DTT, most likely because of reoxidation by traces of Fe(III):EDTA.

3.3. Quantification of electrophiles formed by different oxidizing systems

The amount of Fl-GSH adduction to oxidized protein that was used as an indicator of the formation of electrophiles in the protein was quantified by using a purified and quantified sample of ribonuclease A covalently labeled with NHS-fluorescein. In this way, the amount of Fl-GSH adducted to the oxidized samples could be quantified in relation to the initial concentration of ribonuclease A (Fig. 4B).

All the different oxidizing systems led to the formation of electrophiles in ribonuclease A, evidenced by formation of adducts with Fl-GSH. The highest yield of electrophile in ribonuclease A was given by oxidation with singlet oxygen in D_2O. However, if the amount of adduct formed is normalized against the amount of reactive species produced, then, peroxyl radicals were the most efficient in producing electrophiles in the protein. The lower concentration of AAPH used was expected to

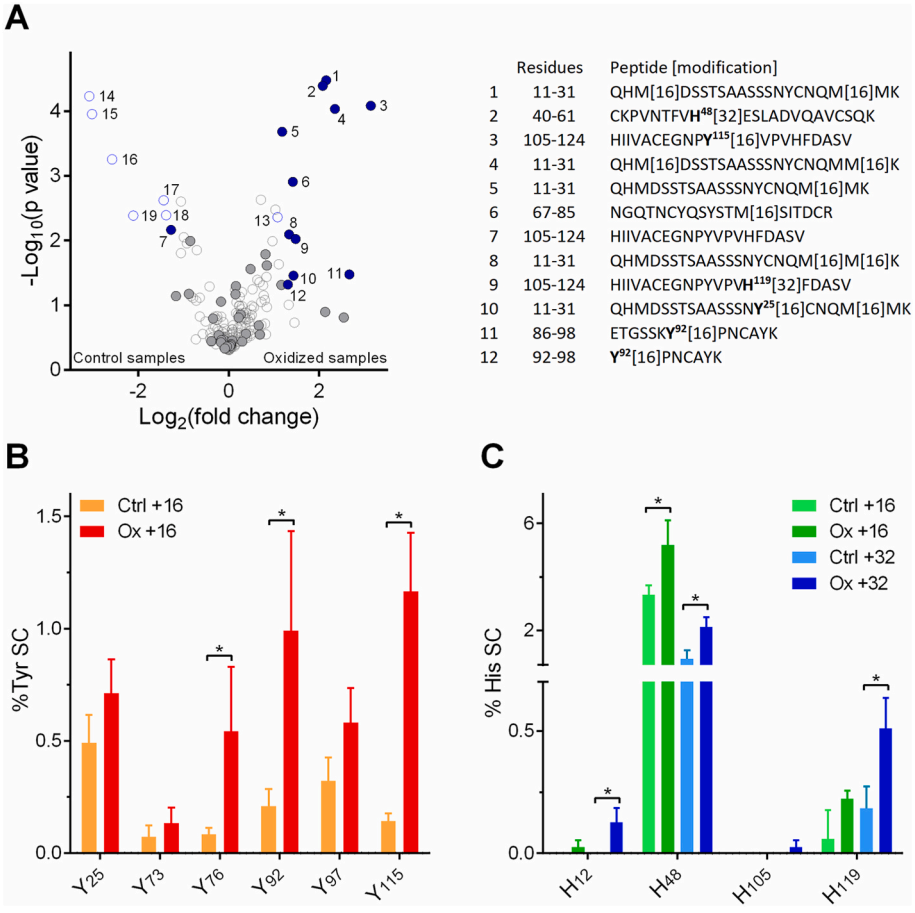
generate 49 μM peroxyl radical in the time assessed, and led to the formation of equivalent amounts of electrophiles to that formed by 300 μM radicals produced by peroxynitrite, or 200 μM ferryl complex from the Fenton-like reaction. This difference is probably because of the different reactivity that leads to different product distribution.

3.4. Identification of the sites of oxidation in ribonuclease A by mass spectrometry

We chose the peroxyl-mediated oxidation of ribonuclease because it had good yields of formation of electrophiles (Fig. 4), it could be performed in a very controlled and reproducible manner, and peroxyl radicals are likely important oxidants in vivo. After oxidation of the protein, we digested it with trypsin and analyzed the resulting peptides by LC-MS/MS. A full coverage of ribonuclease A peptides was observed (Fig. S2). To identify potential residues in the protein that could generate electrophiles, we focused our search on peptides containing Tyr+16, which corresponds to the mass of the HOCHDA residue, and on His+32, which corresponds to the mass of 3-(5-hydroxy-2-oxo-2,5-dihydro-1H-imidazole-5-yl)-L-alanine, and is the other expected electrophile in ribonuclease A. We also included Met+16 and His+16, as other products of oxidation (not expected to be electrophilic). Treating ribonuclease A with AAPH increased peptides with Tyr+16, His+32, Met+16 and His+16 (Fig. 5).

Upon treating ribonuclease A with peroxyl radicals, the sample was enriched in peptides with the specified oxidative modifications, including Tyr+16 and His+32, which are electrophilic candidates. All overrepresented peptides of ribonuclease A in the oxidized samples had at least one oxidative modification. The only underrepresented peptide was peptide 7, HIIVACEGNPYVPVHFDASV, which contains three unmodified target sites (Fig. 5A–Table S1), indicating the loss of this intact peptide by oxidation. Of the 6 tyrosine residues of the protein, Y76, Y92, and Y115 were found to be significantly more mono-oxygenated after exposure to peroxyl radicals than the control (Fig. 5B). The mono-oxygenation of Y115 has been reported previously in ribonuclease A oxidized with peroxyl radicals [32]. Regarding the histidine residues, one of the four residues, H48, showed higher mono-oxygenation relative to control when exposed to peroxyl radicals (Fig. 5B), and three showed higher di-oxygenation, H12, H48 and H119. H12 and H119 show the greatest increase compared to the control. The MS/MS fragmentation spectra of the peptides containing Y76 + 16, Y115 + 16 and H48 + 32 are shown in Fig. 6. A very high coverage of the peptide at high resolution is obtained, supporting the assigned modification with great confidence.

The high levels of modification of Y76, Y92 and Y115 are probably because these residues are very exposed to the solvent and are easily accessible to oxidation by aqueous peroxyl radicals (Fig. 7). H119 is part of the active site along with H12 and K41 and is also very exposed to the solvent (Fig. 7). H48 also shows a significant increase in di-oxygenation compared to the control, and although it is buried in the protein with



	Residues	Peptide [modification]
1	11-31	QHM[16]DSSTSAASSSNYCNQM[16]MK
2	40-61	CKPVNTFV**H**^{48}[32]ESLADVQAVCSQK
3	105-124	HIIVACEGNP**Y**^{115}[16]VPVHFDASV
4	11-31	QHM[16]DSSTSAASSSNYCNQMM[16]K
5	11-31	QHMDSSTSAASSSNYCNQM[16]MK
6	67-85	NGQTNCYQSYSTM[16]SITDCR
7	105-124	HIIVACEGNPYVPVHFDASV
8	11-31	QHMDSSTSAASSSNYCNQM[16]M[16]K
9	105-124	HIIVACEGNPYVPV**H**^{119}[32]FDASV
10	11-31	QHMDSSTSAASSSN**Y**^{25}[16]CNQM[16]MK
11	86-98	ETGSSK**Y**^{92}[16]PNCAYK
12	92-98	**Y**^{92}[16]PNCAYK

Fig. 5. Oxidation of tyrosine and histidine residues in ribonuclease A. A) Volcano plot showing changes in peptide spectral counts between control and oxidized samples. Four replicates were performed for each condition. Each point represents a peptide: filled circles correspond to peptides from ribonuclease A, while hollow circles represent peptides from contaminant proteins. Gray dots represent peptides that do not meet the fold change and/or p-value cutoff thresholds, or do not achieve significance after Benjamini-Hochberg (BH) correction. Blue points correspond to peptides that meet all statistical filters and are considered significantly differentially abundant. **B)** Quantification of oxidized tyrosine residues (Y+16); and **C)** Quantification of oxidized histidine residues (H+16 and H+32). The values were determined as the percentages of spectra with modified amino acids (+16 or +32) relative to the total spectra of ribonuclease A, determined by MS in control and peroxyl radical-oxidized samples. The Mann-Whitney U statistical test was applied, significance levels are indicated as * for $P < 0.05$.

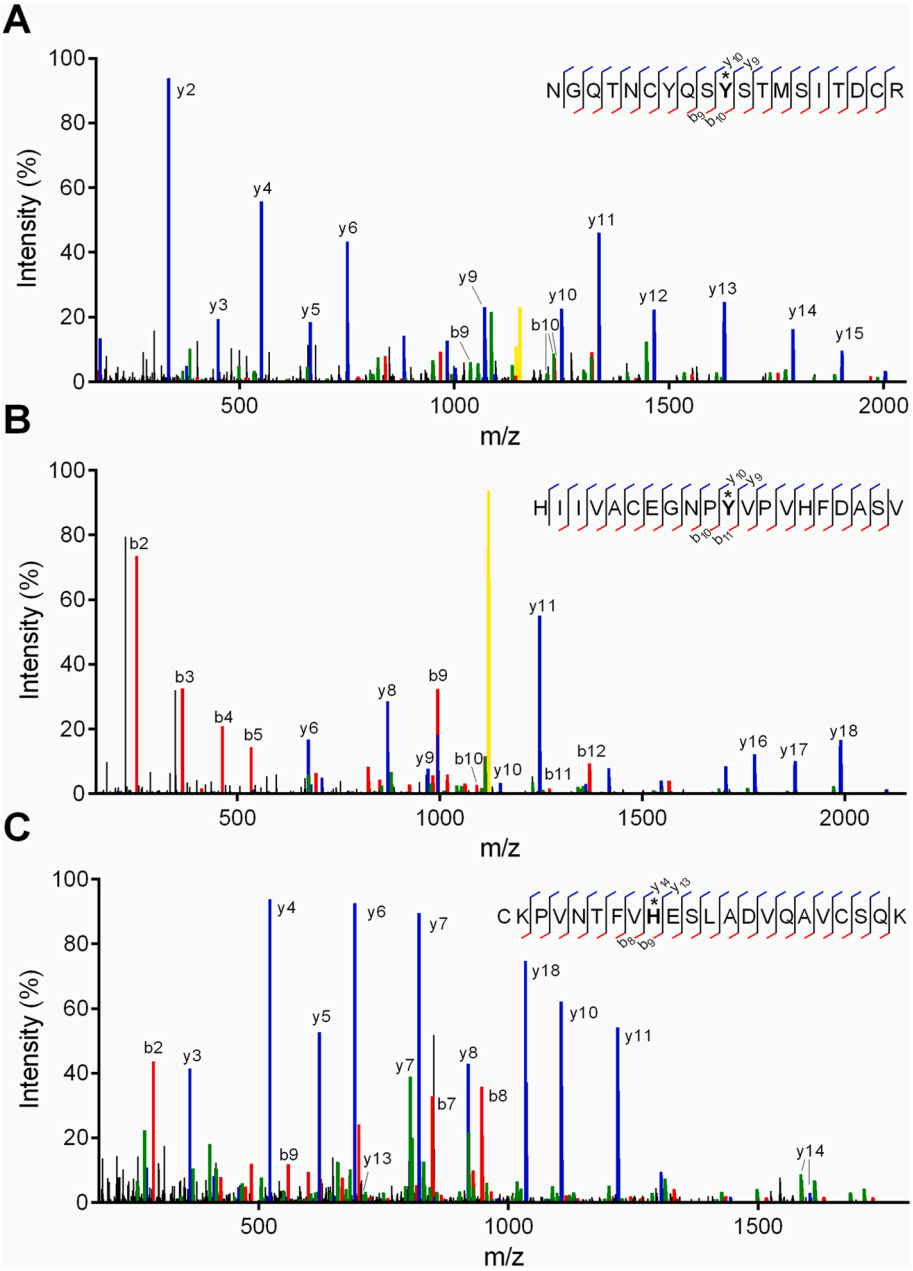


Fig. 6. Representative MS/MS spectra of the main oxidized peptides of ribonuclease A. The yellow, red, blue, and green peaks represent the precursor ion, b-series, y-series, and neutral losses, respectively. **A)** NGQTNCYQSY(+16)STMSITDCR peptide containing Tyr 76 modified by +16. **B)** HIIVACEGNPY(+16)VPVHFDASV peptide containing Tyr 115 modified by +16. **C)** CKPVNTFVH(+32)ESLADVQAVCSQK peptide containing His 48 modified by +32.

less exposure to the solvent, it is located below a flexible loop with high B factor (Fig. S3) that could favor its exposure. Therefore, the best candidates to form electrophilic derivatives in ribonuclease A are Y115, Y92, Y76, H119, H12 and H48, mostly located on the protein surface and exposed to the solvent (Fig. 7).

4. Discussion

The formation of an adduct between Fl-GSH and G-HOCHDA was confirmed by HPLC and mass spectrometry and validated the use of Fl-GSH to study the formation of electrophilic sites in proteins. The oxidation of ribonuclease A by different systems led to the formation of electrophilic centers that reacted with Fl-GSH and the formation of covalent adducts was confirmed by HPLC and SDS-PAGE. The preferred method was gel filtration by HPLC (GF-HPLC) and the measurement included treating labeled ribonuclease A samples with DTT so that formation of mixed disulfides with the protein that could generate false positive signals could be dismissed. Furthermore, GF-HPLC showed that native ribonuclease A was not labeled with Fl-GSH, indicating that an oxidation step is necessary for the formation of electrophiles in the protein.

The reactivity of nucleophiles and electrophiles is often explained by Pearson's hard and soft, acid and bases theory [36], which succinctly indicates that groups that possess more polarizable electrons such as thiolates are softer than less polarizable groups such as amines. Soft nucleophiles will react preferentially with soft electrophiles that also possess more polarizable electrons such as α,β-unsaturated carbonyls rather than with harder electrophiles such as aldehydes [36].Thus, Fl-GSH is expected to react preferentially with soft electrophiles, including HOCHDA and histidine-derived electrophiles.

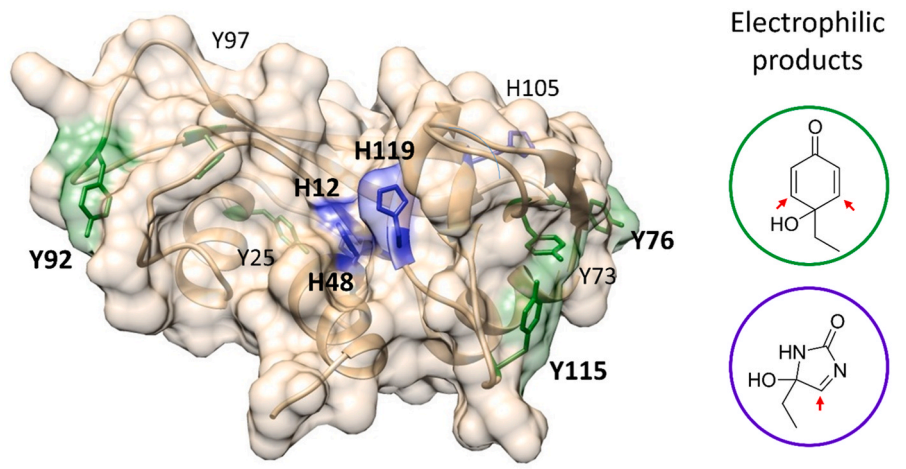


Fig. 7. Proposed electrophilic products from ribonuclease A oxidation and their location. The three-dimensional structure of ribonuclease A is depicted as ribbons and the surface is semitransparent. The histidine and tyrosine residues are shown in blue and green respectively. Most of these residues are on the surface of the protein and could generate the electrophilic products 3-(5-hydroxy-2-oxo-2,5-dihydro-1H-imidazole-5-yl)-L-alanine from histidine (blue circle) and HOCHDA from tyrosine (green circle). The Michael addition sites are indicated by red arrows. Mass spectrometric analysis showed that Y115 generated the most mono-oxygenated product upon exposure to AAPH, followed by Y92, whereas H12 and H119 generated the most di-oxygenated product, followed by H48, suggesting that these residues form electrophilic products upon oxidation.

The reaction between GSH and tyrosine-derived electrophiles has been observed before. Nagy et al. found that the dipeptide tyrosyl-glycine and Myoglobin Tyr-151 formed and adduct with GSH after being exposed to an oxidizing system that led to the formation of tyrosyl radical and superoxide, that ultimately produces HOCHDA and related species [22]. Although they could not unambiguously determine the structure of the products, they showed the formation of glutathionyl adducts by LC-MS. They found that the rate constant for GSH addition to two diasteroisomers of HOCHDA-Gly (or HOHICA-Gly [19]), was $k \sim 0.16\ M^{-1}\ s^{-1}$, and to be reversible ($k_{off} \sim 2 \times 10^{-5}\ s^{-1}$) [22]. Using the same oxidizing system, Das et al. showed that the addition of GSH also occurred with the HOCHDA derivative of tyrosine 14 in oxidized insulin, and both mono and di-GSH adducts could be observed by mass spectrometry [23]. Therefore, the reaction between HOHICA and HOCHDA with GSH is documented and confirms their electrophilic nature.

In this work, the highest yield of electrophile formation in ribonuclease A was given by oxidation with singlet oxygen in D_2O. Although methylene blue is a photosensitizer known to cause both type I and type II oxidations, involving both radical intermediates and singlet oxygen [37], the ten-fold higher yield of electrophiles in deuterated water than in regular water indicates that singlet oxygen is the main species involved in producing electrophiles in the protein. Singlet oxygen decays spontaneously in water to the basal triplet state with a half-life of 3 μs, but in deuterated water this half-life increases to 53 μs [33], resulting in an increased steady state concentration of singlet oxygen. Singlet oxygen is known to react preferentially with cysteine, methionine, histidine, tryptophan and tyrosine. Studies by Leinisch et al. showed that the main targets of singlet oxygen in ribonuclease A were methionine, histidine, tyrosine and proline, and mono-oxygenated products of these residues (M+16) could be identified by mass spectrometry [32]. Furthermore, they observed the formation of covalent dimers, trimers and higher oligomers of ribonuclease A, and identified several cross-linked peptides involving the formation of Tyr-Tyr, Lys-Tyr, His-Arg and His-Lys crosslinks.

Histidine oxidized with singlet oxygen yields endoperoxide intermediates that rearrange to several products including the dioxygenation product (H+32), 3-(5-hydroxy-2-oxo-2,5-dihydro-1H-imidazole-5-yl)alanine (Fig. 7), which is electrophilic and can react with another His, cysteine and lysine to give crosslinking products [37–40]. Other studies showed that chemically modifying histidines in ribonuclease A resulted in decreased oligomerization [41], supporting an important role of histidines as generators of electrophiles. Furthermore, the His-His and His-Lys crosslinks could be partially broken by DTT [39,41], indicating a reversible reaction and supporting the identity of the products as Michael adducts. These products of histidine with singlet oxygen should be differentiated from 2-oxo-histidine (H+16), which has been identified as a product of oxidation of histidine by metal-catalyzed production of hydroxyl radical in LDL, Cu,Zn-SOD, β-amyloid peptide and also in the response factor PerR in *Bacillus subtilis* [42–44], because this product of histidine is not electrophilic and does not react further with thiols or other nucleophiles. Thus, the oxidation product of histidine, 3-(5-hydroxy-2-oxo-2,5-dihydro-1H-imidazole-5-yl)-L-alanine, is likely to be involved in the formation of adducts with Fl-GSH in ribonuclease A.

The reaction of tyrosine with singlet oxygen produces HPOCHDA that can hydrolyze or be reduced to HOCHDA [19,21,37]. In free tyrosine, this HOCHDA product rearranges through an intramolecular Michael addition to yield HOHICA (Fig. 1) [21]. Previous mass spectra analysis of oxidized ribonuclease A showed that tyrosine was oxidized by singlet oxygen to a mono-oxygenated product consistent with HOCHDA [32]. Apparently, this modification is not involved in inter-protein crosslinking in ribonuclease A because no His-HOCHDA or Lys-HOCHDA peptides were observed by mass spectrometry, but this could be because of several reasons, including steric hindrance, a more selective reactivity to softer nucleophiles such as thiols, or a high reversibility that resulted in its loss when samples were prepared with reductants for LC-MS/MS [32]. It is interesting to point out that in that study only a small fraction of total modified tyrosine could be accounted for in the form of Tyr+16 [32], indicating that most tyrosine was modified to unknown products, some of which might be Michael adducts of HOCHDA with the reductant used during sample preparation. The detection of these products is difficult because their structure has to be known beforehand to be positively identified by mass spectrometry analysis, as de novo analysis of chemical structures from a complex mixture by mass spectrometry remains challenging [45].

Peroxyl radicals also generated electrophiles in ribonuclease A. It is plausible that tyrosine reacts with the peroxyl radicals derived from the azo-initiator ($k = 4.8 \times 10^3\ M^{-1}s^{-1}$ [46]), and the resulting tyrosyl radical reacts with another peroxyl radical generating a LPOCHDA-like product [11], with a similar reactivity to that of HOCHDA. Previous mass spectra studies of ribonuclease A oxidized by AAPH showed that the main modifications occurred on methionine, histidine, tyrosine and

proline, the same as with singlet oxygen, and mono-oxygenated products of these residues could be identified and also dityrosine, suggesting the formation of HOCHDA [32]. In this work, we identified mono-oxygenated tyrosine residues, Y115, Y92, and Y76, that are exposed to the solvent and are very good candidates to be making HOCHDA. The formation of the isomer dihydroxyphenylalanine cannot be discarded based on MS alone, but previous experiments showed that peroxyl radicals generate exclusively HOCHDA-type products [11]. The formation of di-oxygenated products of histidine, in H119, H12 and H48, also suggest formation of 3-(5-hydroxy-2-oxo-2,5-dihydro-1H-imidazole-5-yl)alanine, that is electrophilic, but further experiments are needed to confirm their identity.

Hydroxyl radical and ferryl complex formed in Fenton-like reactions have been shown to form reactive aldehydes from the oxidation of lysine, arginine, proline and the peptide backbone [3]. However, these are hard electrophiles that will react preferentially with hard nucleophiles such as lysines but not with thiols, that are soft nucleophiles [47], thus aldehydes are not expected to be detected by Fl-GSH. Aldehydes can react with cysteine to yield a stable cyclic thiazolidine, but this depends on having a free amine group that starts the reaction [48,49], which is not present in Fl-GSH. For the same reason, we cannot rule out the possible reaction between Fl-GSH and Schiff bases, that would give a thioethylamine. The reaction of hydroxyl radical or ferryl complex with tyrosine also yields HOCHDA [19,30], and may yield electrophiles from histidine, and these are the most likely candidates for the electrophilic products detected on ribonuclease A oxidized using the Fenton-like system.

Oxidation of proteins by peroxynitrite was not known to generate electrophilic products. In the absence of carbon dioxide, peroxynitrite homolyzes to hydroxyl radical and nitrogen dioxide that could explain the formation of similar products to those observed by hydroxyl radical [9]. Nitrogen dioxide is expected to generate nitration products by termination reactions with protein radicals, in particular tyrosyl radicals [2]. No path to an electrophilic product is known, but could involve a minor path where the nitrogen dioxide added to the C1 in the tyrosyl radical, followed by hydrolysis leading to HOCHDA and nitrite. In the presence of carbon dioxide, carbonate radical is generated instead of hydroxyl radical. Carbonate radical is a potent one electron oxidant, generating tyrosyl radical efficiently, but no route to electrophilic products from proteins is known yet [10]. However, the reaction of carbonate radical with the spin trap DMPO was observed to generate an unstable adduct that is hydrolyzed by water, ultimately yielding DMPO-OH, the same product that is generated in the reaction of DMPO with hydroxyl radical [50]. It could be that a minor path involves the formation of a transient adduct of carbonate radical with tyrosine (or tyrosyl radical) that is hydrolyzed to HOCHDA. Because HOCHDA is not aromatic, it is more polar, and may be formed in low yields in the reaction of tyrosine with carbonate radical, it could have escaped previous detection. Regardless of the mechanism, this work is the first to report the formation of electrophilic products from protein oxidation by peroxynitrite in the absence and presence of carbon dioxide and warrants further study.

It was very interesting to find that all the biologically relevant oxidants tested yielded electrophiles in proteins. It suggests that formation of electrophilic residues in proteins may be a widespread event resulting from common oxidations that include not only those involving the formation of tyrosine hydroperoxide from the reaction of tyrosyl radical with superoxide [22,23] but also those shown here, caused by singlet oxygen, peroxyl radicals, hydroxyl radical and peroxynitrite.

The formation of electrophiles from free radical oxidations is most often associated with lipid oxidation products, such as 4-hydroxynonenal (HNE), malondialdehyde, 15DpgJ2, neuroprostanes or nitrolipids [5,27,51–53]. These lipid-derived electrophiles have been shown to react with glutathione and protein thiols, as well as with histidine, lysine and DNA bases [27,52,54]. There are multiple cellular responses to these electrophiles, ranging from activation of the antioxidant response through Keap1-Nrf2, cell survival carrying carcinogenic DNA modifications, to cell death, depending on the concentration and nature of the electrophile [55,56]. It is plausible then that mildly oxidized proteins containing electrophiles could also trigger these responses. For instance, upon oxidation of proteins, their derived electrophiles could react with Keap1, release Nrf2 and trigger the antioxidant response [55]. In line with this, tert-butylhydroquinone is a food additive that shares structural similarity to tyrosine and has been observed to trigger the antioxidant response via Nrf2 after it is oxidized to the benzoquinone [57]. Though it is likely that the benzoquinone is significantly more reactive to thiols than HOCHDA, the structural resemblance suggests that HOCHDA may also trigger the antioxidant response in cells. It is also plausible that beyond a certain threshold these modifications could contribute to protein aggregation, through inter-protein cross-linking, that may accumulate over time and contribute to protein denaturation and aggregation, such as those observed in aging and neurodegenerative diseases [5,6,58,59]. To explore these hypotheses, we need to further characterize these electrophilic residues in proteins, for which we need new tools such as nucleophilic tags that react more rapidly and favorably than Fl-GSH, to enable us to identify these modifications in vivo and assess their biological relevance.

In summary, this work showed that: (a) fluorescein-labeled glutathione (Fl-GSH) reacts with the tyrosine-derived electrophile HOCHDA; (b) singlet oxygen oxidation of ribonuclease A generates electrophilic residues detectable and quantifiable via Fl-GSH and gel filtration-HPLC; (c) biologically relevant oxidants—including peroxyl radicals, peroxynitrite (with or without CO_2), and ferryl (from Fe(II):EDTA and H_2O_2)—also produce electrophiles in ribonuclease A; and (d) mass spectrometry identified potential electrophilic sites, including mono-oxygenated tyrosines (Y76, Y92, Y115) and dioxygenated histidines (H12, H48, H119). These findings underscore the formation of protein electrophiles as relevant oxidation products.

CRediT authorship contribution statement

Ana C. Lopez: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Silvina Acosta:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Mauricio Mastrogiovanni:** Writing – review & editing, Validation, Investigation, Formal analysis. **Williams Porcal:** Writing – review & editing, Methodology, Investigation. **María Magdalena Portela:** Investigation, Formal analysis. **Rosario Durán:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation. **Rafael Radi:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition. **Ana Denicola:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Matias N. Möller:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rbc.2025.100048.

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