Oxidation of hypotaurine and cysteine sulphinic acid by peroxynitrite

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Peroxynitrite mediates the oxidation of the sulphinic group of both HTAU (hypotaurine) and CSA (cysteine sulphinic acid), producing the respective sulphonates, TAU (taurine) and CA (cysteic acid). The reaction is associated with extensive oxygen uptake, suggesting that HTAU and CSA are oxidized by the one-electron transfer mechanism to sulphonyl radicals, which may initiate an oxygen-dependent radical chain reaction with the sulphonates as final products. Besides the one-electron mechanism, HTAU and CSA can be oxidized by the two-electron pathway, leading directly to sulphonate formation without oxygen consumption. The apparent second-order rate constants for the direct reaction of peroxynitrite with HTAU and CSA at pH 7.4 and 25 °C are 77.4 ± 5 and $76.4 \pm 9 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. For both sulphinates, the apparent second-order rate constants increase sharply with decrease

in pH, and the sigmoidal curves obtained are consistent with peroxynitrous acid as the species responsible for sulphinate oxidation. The kinetic data, together with changes in oxygen uptake, sulphinate depletion, sulphonate production, and product distribution of nitrite and nitrate, suggest that oxidation of sulphinates by peroxynitrite may take place by the two reaction pathways whose relative importance depends on reagent concentrations and pH value. In the presence of bicarbonate, the direct reaction of sulphinates with peroxynitrite is inhibited and the oxidative reaction probably involves only the radicals $\cdot\text{NO}_2$ and $\text{CO}_3^{\cdot-}$, generated by decomposition of the peroxynitrite- CO_2 adduct.

Key words: cysteine sulphinic acid, hypotaurine, peroxynitrite, reactive sulphur species, sulphonyl radical, taurine.

INTRODUCTION

CSA (cysteine sulphinic acid) and HTAU (hypotaurine; also known as 2-aminoethanesulphinic acid) are precursors of TAU (taurine; also known as 2-aminoethanesulphonic acid), the main end product of cysteine metabolism in mammals [1]. Although the enzymatic routes leading to CSA and HTAU have been well characterized [2], the search for a specific enzymatic system capable of oxidizing the sulphinic group of both compounds to the respective sulphonate has been unsuccessful. Only non-specific oxidizing agents such as UV irradiation [3], hydroxyl radicals [4] and photochemically generated singlet oxygen [5] have been found to accomplish this oxidation *in vitro*. These findings have been related to the proposed role of HTAU as an antioxidant and free radical-trapping agent *in vivo* [6]. In agreement with this are reports that HTAU inhibits lipid peroxidation [7], prevents the inactivation of superoxide dismutase by H_2O_2 [8] and quenches the oxidants released by human neutrophils [9]. It has also been reported that HTAU can act as a protective agent against oxidative damage in the male reproductive tract and in regenerating liver, tissues in which HTAU is present in millimolar concentrations [10–12]. Furthermore, it has been proposed that the high levels of TAU found in tissues or cells, such as sperm, neutrophils and retinal tissue [13–15], reflect the turnover of HTAU by oxidative reactions and might be viewed as an indirect measure of the oxidative stress within these tissues.

Peroxynitrite, which is formed *in vivo* by the diffusion-controlled reaction between nitric oxide ($\cdot\text{NO}$) and superoxide anion ($\text{O}_2^{\cdot-}$), has been receiving increasing attention as a mediator of various human diseases, including neurodegenerative disorders, chronic inflammation, autoimmune diseases, ischaemia-reperfusion injury and septic shock [16]. Peroxynitrite is a strong oxidizing and nitrating agent and reacts with various biomolecules, such as lipids, thiols, amino acids, antioxidants and nucleic

acids [17–24]. At physiological pH, both the peroxynitrite anion (ONO_2^-) and its conjugate acid (ONO_2H ; $\text{p}K_a = 6.8$) are present. Whereas ONO_2^- is a relatively stable species, ONO_2H decays rapidly with a half-life of $<1 \text{ s}$ at pH 7.4 and 37 °C. [Unless otherwise specified, throughout this paper, peroxynitrite refers both to peroxynitrite anion (ONO_2^-) and to peroxynitrous acid (ONO_2H)]. Peroxynitrite decomposition produces nitrate, nitrite and oxygen with yields that are dependent on the pH, concentration of peroxynitrite and temperature [25]. It has been reported that peroxynitrite can oxidize suitable substrates, either through a direct one- or two-electron mechanism or by an indirect one-electron reaction involving $\cdot\text{OH}$ and $\cdot\text{NO}_2$ radicals released during peroxynitrite homolysis [26–32].

Under physiological conditions, peroxynitrite predominantly reacts with CO_2 to give a short-lived peroxynitrite- CO_2 adduct, which decomposes to CO_2 and nitrate before it can react with biological targets [33–36]. It is now widely recognized that the oxidation and nitration of many biological species that take place when both peroxynitrite and CO_2 are present involve the radicals $\text{CO}_3^{\cdot-}$ and $\cdot\text{NO}_2$ generated during the decomposition of the adduct [36–40].

Among target molecules that react with peroxynitrite, sulphur-containing compounds such as thiols and methionine have been extensively investigated [18,23,41–44]. However, the reaction of peroxynitrite with compounds containing sulphur in higher oxidation states such as sulphinates (RSO_2^-) has been only partially characterized [45].

Recently, we showed that the sulphinates, HTAU and CSA are capable of preventing peroxynitrite-mediated reactions such as tyrosine nitration, α_1 -antiproteinase inactivation and low-density lipoprotein oxidative modification [46]. In the present study, we examine peroxynitrite-mediated oxidation of HTAU and CSA by combining kinetic, oxygen consumption and product identification studies. The results demonstrate that HTAU and CSA

Abbreviations used: CA, cysteic acid; CSA, cysteine sulphinic acid; DMPO, 5,5-dimethylpyrrolidine-N-oxide; DTPA, diethylenetriaminepenta-acetic acid; HTAU, hypotaurine; TAU, taurine.

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are oxidized by peroxynitrite to form the corresponding sulphonates, TAU and CA (cysteic acid). The reaction may occur either through one- or two-electron mechanisms whose relative importance depends on reagent concentrations and pH. We propose that the oxidation of sulphinates by the one-electron pathway produces sulphonyl radicals as intermediates. Sulphonyl radicals, in turn, are involved in oxygen-dependent chain reactions by which sulphinates are oxidized to sulphonates. The effect of bicarbonate/CO₂ on peroxynitrite-mediated oxidation of sulphinates is also evaluated.

MATERIALS AND METHODS

Materials

CSA, CA, HTAU, TAU and DMPO (5,5-dimethylpyrroline-*N*-oxide) were obtained from Sigma. DTPA (diethylenetriamine-penta-acetic acid), tetrabutylammonium bisulphate, *o*-phthalaldehyde and manganese dioxide were obtained from Fluka (Gillingham, Dorset, U.K.). All other reagents were of the highest purity commercially available.

Peroxynitrite was synthesized from potassium nitrite and H₂O₂ under acidic conditions as described previously [47], and excess H₂O₂ was removed by treatment with granular manganese dioxide. Typical peroxynitrite concentrations after freeze fractionation were in the range 600–700 mM as determined by measuring the absorbance A_{302} using a molar absorption coefficient of 1670 M⁻¹ · cm⁻¹. Stock solutions of peroxynitrite were diluted with 0.1 M NaOH immediately before use to achieve the desired concentration.

Reaction of HTAU or CSA with peroxynitrite

The reaction mixture contained HTAU or CSA at appropriate concentrations in 0.2 M phosphate buffer at pH 7.4 or 5.5, in the absence or presence of 25 mM sodium bicarbonate. To avoid metal-catalysed oxidative reactions, all samples contained 0.1 mM DTPA. The reaction was started by adding peroxynitrite at a final concentration of 0.2 mM. To control the non-specific effects of contaminating substances present in the peroxynitrite solutions or stable peroxynitrite-decomposition products (nitrite and nitrate), peroxynitrite was first incubated in phosphate buffer/DTPA for 10 min before the addition of HTAU or CSA (reverse-order addition).

Oxygen uptake

Oxygen uptake was performed using a Gilson 5/6 oxygraph and measured with a Clark-type electrode in a water-jacketed cell (1.8 ml) at 25°C. The saturation oxygen concentration at this temperature was taken as 235 µM.

HPLC analysis

The amounts of HTAU, CSA, TAU and CA were determined by HPLC using the *o*-phthalaldehyde reagent [48]. Analyses were performed with a Waters chromatograph equipped with a PerkinElmer model LS-1 LC fluorescence detector using a 340 nm filter for excitation, with an emission wavelength of 450 nm. We used a 250 mm × 4.6 mm inner diameter, Symmetry C18, 5 µm column (Waters, Milford, MA, U.S.A.). The mobile phases were: (A) 0.05 M sodium acetate (pH 5.5)/methanol (80:20, v/v) and (B) 0.05 M sodium acetate (pH 5.5)/methanol (20:80, v/v). The elution gradient was as follows: linear from A to 50% B in 5 min followed by isocratic at 50% B. Flow rate: 1 ml/min

at room temperature (20°C). The elution times of CA, CSA, TAU and HTAU were 7.5, 10.5, 24.5 and 26 min respectively.

Nitrite and nitrate were analysed by ion-pairing HPLC as described previously [49]. Samples were injected on to a 250 mm × 4.6 mm inner diameter, Atlantis C18, 5 µm column (Waters), isocratically running at a flow rate of 1 ml/min with 10 mM K₂HPO₄, 10 mM tetrabutylammonium bisulphate in water/acetonitrile (95:5, v/v, pH 7). Detection was at 210 nm using a Waters 996 photodiode array detector. The elution times of nitrite and nitrate were 8.5 and 20.5 min respectively.

Other analyses

Inorganic sulphate was determined by the method of Dodgson [50].

Kinetic experiments

Peroxynitrite decomposition in the presence of HTAU and CSA was studied by stopped-flow absorbance spectroscopy at 302 nm using an Applied Photophysics apparatus (Leatherhead, U.K.) with a mixing time of < 4 ms. Reactions were performed at 25°C by mixing equal volumes of a solution of peroxynitrite, diluted immediately before use to 0.2 mM with 0.01 M NaOH, with solutions of the sulphinates in 0.5 M phosphate buffer containing 0.2 mM DTPA. Sulphinate concentrations were at least 50 times higher than that of peroxynitrite to ensure pseudo-first-order conditions. The final pH of the mixtures was measured at the outlet. Apparent pseudo-first-order rate constants for peroxynitrite decompositions, k_{obs} (s⁻¹) were determined by fitting stopped-flow data to a single exponential decay with a floating end-point. Results from at least four separate determinations were averaged to obtain each value of k_{obs} . The values of k_{app} were determined from the slopes of the plots of k_{obs} versus sulphinate concentrations.

Statistical analysis

Results are expressed as means ± S.E.M. for at least three separate experiments. Graphics and data analysis were performed using GraphPAD Prism 4 software.

RESULTS

Peroxynitrite-mediated oxidation of HTAU and CSA

We evaluated the oxidation of HTAU and CSA by peroxynitrite by monitoring oxygen consumption and the production of corresponding sulphonates, TAU and CA.

As reported previously, decomposition of peroxynitrite results in a pH-dependent O₂ evolution [51,52]. Under our experimental conditions, 200 µM peroxynitrite in 0.2 M phosphate buffer (pH 7.4) released approx. 12 µM O₂. However, in the presence of sulphinates, a fast oxygen consumption was observed (Figure 1); no O₂ uptake occurred in the absence of peroxynitrite or on the addition of peroxynitrite previously decomposed in phosphate buffer (reverse-order addition of peroxynitrite). These results suggest that the peroxynitrite-mediated oxidation of sulphinates generates intermediate free radicals that react with oxygen. In agreement with this, addition of the spin trap DMPO (80 mM) inhibited O₂ consumption, possibly by scavenging the radicals formed before their reaction with oxygen. Figure 2 shows that the addition of 200 µM peroxynitrite to various concentrations of sulphinates (from 0.025 to 20 mM) at pH 7.4 caused a concentration-dependent increase of O₂ uptake, which levelled off at approx. 5 mM sulphinates. HPLC analyses of the incubation mixtures at the end of the reaction showed that HTAU and CSA

Figure 1 Oxygen consumption during the reaction of peroxynitrite with HTAU or CSA: effect of spin trap

Peroxynitrite (200 μ M) was added to the oxygraph chamber at 25 $^{\circ}$ C, containing 1 mM HTAU (A) or 1 mM CSA (B) in 0.2 M phosphate buffer, including 0.1 mM DTPA (pH 7.4). O_2 uptake traces in the absence (●) and presence of 80 mM DMPO (○). The arrows indicate the time points of addition of peroxynitrite.

are oxidized to the corresponding sulphonates, TAU and CA, with yields increasing in a sulphinate concentration-dependent manner (Figure 2). At HTAU concentrations above 1 mM, the amount of TAU produced is higher than the concentration of peroxynitrite added, suggesting the occurrence of reactions that cause an amplification of the oxidation. The amount of oxygen consumed per mol of sulphonate produced decreases considerably with increase in sulphinate concentration, indicating that the reaction proceeds with different mechanisms depending on the sulphinate/peroxynitrite molar ratio.

Effect of pH on the interaction of peroxynitrite with sulphonates

To evaluate the effect of pH on peroxynitrite-mediated oxidation of sulphonates, we monitored HTAU and CSA oxidation at pH 7.4 and 5.5 by sulphonate production, sulphonate depletion and oxygen consumption. The data presented in Figure 3(A) show that, in the reaction of 1 mM HTAU with 200 μ M peroxynitrite at pH 7.4, the value of TAU produced is approx. 90 % of depleted HTAU. Oxygen uptake measurements associated with the reaction revealed a stoichiometry of approx. 0.6 mol of O_2 consumed/mol of HTAU depleted. At pH 5.5, the TAU produced increases by about two times than at pH 7.4 and a stoichiometry of 0.45 mol of O_2 consumed/mol of HTAU depleted was obtained.

Figure 3(B) shows that, in the oxidation of CSA (1 mM) by peroxynitrite (200 μ M) at pH 7.4, CA production accounts for approx. 30 % of the CSA depleted and that approx. 0.5 mol of oxygen is consumed/mol of CSA depleted. Since CA is stable to further oxidation by peroxynitrite (results not shown), these findings indicate that, in the interaction of CSA with peroxynitrite at pH 7.4, a fraction of CSA is oxidized through secondary oxygen-dependent reactions to other unidentified products. Interestingly, at pH 5.5, the product of the interaction of CSA with peroxynitrite is mainly CA ($\sim$ 90 %) and the O_2 consumption associated with the reaction revealed a stoichiometry of approx. 0.5 mol of O_2 uptake/mol of CSA depleted.

Detection of sulphate

Recently, we reported that Methylene Blue-photosensitized oxidation of CSA produces CA in a yield close to 50 % of the CSA depleted [53]. We found that the remaining CSA was degraded to acetaldehyde with release of ammonia, CO_2 and SO_2 . Oxidation of aqueous SO_2 accounted for the excess of oxygen consumption

Figure 2 Oxidation of HTAU and CSA by peroxynitrite as a function of sulphinate concentration

Peroxynitrite (200 μ M) was added to the oxygraph chamber at 25 $^{\circ}$ C, containing different concentrations of HTAU (A) and CSA (B) in 0.2 M phosphate buffer and 0.1 mM DTPA (pH 7.4), and the O_2 consumption was recorded. The reaction mixtures were subsequently analysed for TAU and CA formation by HPLC as described in the Materials and methods section. Insets: enlargement of the lowest sulphonate concentrations.

that accompanied the photochemical reaction and for the detection of sulphate among the oxidation products.

To verify whether CSA undergoes the same degradation process during the reaction with peroxynitrite, we looked for a possible formation of sulphate. We found that, in the reaction of 1 mM CSA with 200 μ M peroxynitrite at pH 7.4, where the recovery of CA was only 30 % of the CSA depleted, 96.5 ± 5 μ M sulphate was produced. Thus, at pH 7.4, the main part of the CSA undergoes degradation, and the formation of sulphate may contribute to the O_2 uptake observed during the reaction. In the oxidation of 1 mM HTAU with 200 μ M peroxynitrite at pH 7.4, where the yield of TAU is roughly similar to the value of HTAU depleted, only 16.5 ± 2 μ M sulphate was detected.

Kinetics of the reaction between peroxynitrite and sulphonates

We studied the decay of peroxynitrite (0.1 mM) in the presence of 50–250-fold excess of HTAU or CSA from pH 5.5 to 8.5, at 25 $^{\circ}$ C. At a given pH, the observed pseudo-first-order rate constant

Figure 3 Oxidation of HTAU and CSA by peroxynitrite: effect of pH

Peroxynitrite ($200 \mu\text{M}$) was added to the oxygraph chamber at 25°C , containing 1 mM HTAU (A) and 1 mM CSA (B) in 0.2 M phosphate buffer and 0.1 mM DTPA, at pH 7.4 and 5.5, and the O_2 consumption was recorded. The reaction mixtures were subsequently analysed for sulphinate depletion and sulphonate production by HPLC.

(k_{obs}) of peroxynitrite decomposition increases linearly with the concentration of HTAU and of CSA (Figure 4). As shown in the insets to Figure 4, for both sulphinates, the apparent second-order rate constant (k_{app}) is pH-dependent: at pH 7.4, the values are 77.4 ± 5 and $76.4 \pm 9 \text{ M}^{-1} \cdot \text{s}^{-1}$ for HTAU and CSA respectively. At pH 5.5, these values increase to 379 ± 7 and $234 \pm 5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Also, the pH profiles display a sigmoidal shape with inflection points at pH 6.7 for HTAU and pH 6.9 for CSA, consistent with ONO_2H ($\text{pK}_a = 6.8$) being the species responsible for sulphinolate oxidation. The sulphinic groups of HTAU and CSA, with pK_a values of 1.5 and 2.5 respectively [54], react in the anionic forms within the pH range studied here.

Measurement of nitrite and nitrate during the oxidation of sulphinates by peroxynitrite

The concentrations of nitrite and nitrate formed during the decomposition of peroxynitrite at pH 7.4, in the presence of various concentrations of sulphinates, are shown in Figure 5. As reported previously [25,49], decomposition of peroxynitrite yields nitrate as a major product and nitrite as a minor product. However, in the presence of sulphinates, the concentration of nitrate decreased with a concomitant increase in nitrite. Nitrite and nitrate yields accounted for all the reacted peroxynitrite, indicating that peroxynitrite oxidizes sulphinates to sulphonates, forming nitrite as a major product. Figure 5 (insets) shows the pH dependence of the yields of nitrite obtained by reacting $200 \mu\text{M}$ peroxynitrite with 20 mM HTAU or CSA. For both sulphinates, the production of nitrite decreased with increase in pH.

Effect of bicarbonate on peroxynitrite-mediated oxidation of sulphinates

As reported, in biological environments, the reactivity of peroxynitrite is mediated by its fast reaction with CO_2 [33–35]. To

Figure 4 Kinetics of the reaction of peroxynitrite with HTAU and CSA

Peroxynitrite (final concentration $100 \mu\text{M}$) was mixed with different concentrations of HTAU (A) or CSA (B) in 0.25 M phosphate buffer containing 0.1 mM DTPA, at different pH values and 25°C , and the k_{obs} for peroxynitrite decay at 302 nm was determined. Each data point represents the average of at least four separate determinations with S.D. value below 8%. Insets: apparent second-order rate constants (k_{app}) for the reaction between peroxynitrite and HTAU and CSA as a function of pH. The values of k_{app} were determined from the slopes of the plots of k_{obs} versus [HTAU] and [CSA].

evaluate the effects of $\text{HCO}_3^-/\text{CO}_2$ on peroxynitrite-mediated oxidation of sulphinates, we monitored HTAU and CSA oxidation at pH 7.4 and 5.5, in the presence of 25 mM bicarbonate, by sulphonate production, sulphinolate depletion and oxygen consumption (Figure 6). Compared with the results obtained in the absence of bicarbonate (Figure 3), in the presence of added NaHCO_3 , depletion of sulphinates is inhibited either at pH 7.4 ($\sim 35\%$ inhibition) or pH 5.5 ($\sim 60\%$ inhibition), but the resulting stoichiometry i.e. mol of oxygen uptake/mol of depleted sulphinolate, is only marginally affected. The oxidation of CSA at pH 7.4 in the presence of NaHCO_3 produces CA in a yield close to 30% of the CSA depleted, again indicating that CSA is partially degraded to other products.

DISCUSSION

The results demonstrate that the sulphinates (RSO_2^-), HTAU and CSA, are oxidized by peroxynitrite to form the corresponding sulphonates (RSO_3^-), TAU and CA. The reaction is accompanied by extensive oxygen uptake, suggesting that the peroxynitrite-mediated oxidation of sulphinates involves an initial one-electron transfer mechanism, generating intermediate radicals that quickly react with oxygen. It is now widely recognized that one-electron oxidation is not a direct reaction of peroxynitrite but involves $\cdot\text{NO}_2$ and $\cdot\text{OH}$ released during the degradation of peroxynitrite

Figure 5 Oxidation of HTAU and CSA by peroxynitrite: nitrite and nitrate formation

Peroxynitrite (200 μM) was incubated with different concentrations of HTAU (**A**) and CSA (**B**) in 0.2 M phosphate buffer (pH 7.4), containing 0.1 mM DTPA at room temperature (25°C) for 15 min, followed by the determination of NO_2^- and NO_3^- as described in the Materials and methods section. Values were corrected for contaminant concentrations of nitrite and nitrate present in the peroxynitrite solution [$\sim 40 \mu\text{M}$ NO_2^- and $60 \mu\text{M}$ NO_3^- in 200 μM peroxynitrite, calculated by allowing the spontaneous decomposition of peroxynitrite in 0.2 M phosphate buffer (pH 3), and by assuming that under these conditions all peroxynitrite decomposed to NO_3^-]. Insets: nitrite formation as a function of pH after incubation of 20 mM HTAU (**A**) or 20 mM CSA (**B**) with 200 μM peroxynitrite.

[30–32,52,55,56]. Accordingly, the sulphinates can be indirectly oxidized to sulphonyl radicals ($\text{RSO}_2^\bullet$) as in reaction 1:

The consumption of oxygen by the peroxynitrite-dependent oxidation of sulphinates could result from the known reaction of sulphonyl radicals with oxygen, producing sulphonyl peroxy radical [57] (reaction 2):

The sulphonyl peroxy radical is a highly reactive intermediate [57] and its reaction with excess sulphinate would give the peroxysulphonate (reaction 3):

Figure 6 Effect of added bicarbonate on the oxidation of HTAU and CSA by peroxynitrite

Peroxynitrite (200 μM) was added to the oxygraph chamber at 25°C, containing 1 mM HTAU (**A**) and 1 mM CSA (**B**) in 0.2 M phosphate buffer and 0.1 mM DTPA, in the presence of 25 mM sodium bicarbonate at pH 7.4 and 5.5, and the O_2 consumption was recorded. The reaction mixtures were subsequently analysed for sulphinates and sulphonates by HPLC.

The peroxysulphonate formed would decompose to yield sulphonate and molecular oxygen (reaction 4):

or oxidize the sulphinate present to the sulphonate (reaction 5):

Oxidation of sulphinates through the above reactions accounts for a stoichiometry of 0.5 mol of oxygen consumed/mol of depleted sulphinate, according to the overall reaction:

It should be noted that sulphonyl radicals, through reactions (2) and (3), can initiate an oxygen-dependent radical chain propagation step that could be responsible for an amplification of the oxidation. Indeed, this chain reaction mechanism is evident in the oxidation of HTAU, where, under certain experimental conditions, the amount of TAU produced is higher than the concentration of peroxynitrite added (Figure 2A, at [HTAU] > 1 mM and Figure 3A, pH 5.5).

Another possible reaction previously suggested for the sulphonyl radical [4,58] is its dimerization to form the corresponding disulphone (reaction 7):

Subsequent hydrolysis of the disulphone (reaction 8) could represent an additional route for the production of sulphonates:

However, the production of sulphonates through the intermediate formation of disulphone does not require oxygen, in contrast with the observed oxygen consumption associated with the oxidative reaction (Figure 1).

Sulphonyl radicals have been previously observed during the oxidation of CSA by horseradish peroxidase in the presence of H_2O_2 [59]. It was also reported that the CSA-derived sulphonyl radical can undergo decomposition, producing sulphur dioxide and a carbon-centred radical (reaction 9):

Subsequent oxidation of sulphite (aqueous sulphur dioxide) to sulphate involves additional free radical mechanisms leading to oxygen consumption [60,61]. Detection of sulphate in the incubation mixtures of CSA with peroxynitrite indicates that a CSA-derived sulphonyl radical undergoes a significant decomposition when the oxidative reaction is performed at pH 7.4. Under these conditions, the yield of CA is only 30 % of depleted CSA (Figure 3B), and the oxygen uptake exceeding that required for CA production can account for the oxidation of sulphite to sulphate. Compared with the CSA-derived sulphonyl radical, the sulphonyl radical derived from the oxidation of HTAU appears to have a much lower tendency to decompose, since the yield of TAU was close to 90 % of the depleted HTAU (Figure 3A).

The oxidative reactions mediated by peroxynitrite are expected to take place by both one- and two-electron mechanisms [26–28]. The latter mechanism could represent an additional route of oxidation of sulphinates leading to direct sulphonate formation without oxygen consumption (reaction 10):

Accordingly, the kinetic experiments (Figure 4) indicate that, at pH 7.4, HTAU and CSA react directly with peroxynitrite with apparent second-order rate constants of 77.4 ± 5 and of $76.4 \pm 9 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. For both sulphinates, k_{app} increases sharply with decreasing pH and the sigmoidal curves obtained are consistent with peroxynitrous acid ($\text{p}K_{\text{a}} = 6.8$) as the reactive species.

In agreement with the kinetic data, the O_2 uptake as a function of sulphinate concentration observed in Figure 2 indicates that oxidation of sulphinates by peroxynitrite may occur through the two reaction pathways; their relative importance depends on reagent concentrations. At low RSO_2^- concentrations, where the O_2 uptake associated with the oxidation of sulphinates is higher, the one-electron mechanism with the intermediate formation of sulphonyl radicals probably predominates. At higher RSO_2^- concentrations, the O_2 uptake associated with the oxidation of sulphinates decreases, since the direct reaction of the two sulphinates with peroxynitrite becomes more significant.

In a two-electron process (reaction 10), nitrite and sulphonate would be the only products. If sulphinates are oxidized by a one-electron mechanism (reaction 1), nitrate will also be formed. This is because the peroxynitrite-derived radicals $\bullet\text{OH}$ and $\bullet\text{NO}_2$, initially formed in a solvent cage, undergo rapid recombination to form NO_3^- (~70 %) or escape the cage (~30 %) to give free radicals that react with sulphinates [62,63]. The increase in nitrite and the decrease in the nitrate formed during the reaction of 200 μM peroxynitrite with increasing concentrations

of sulphinates (Figure 5) support the conclusion that the two pathways coexist and that the direct reaction would prevail quantitatively over the one-electron oxidation when sulphinates are present in large excess compared with peroxynitrite. Since the two competing pathways should have similar pH dependences, the finding that the nitrite production decreases with increase in pH could be explained by the occurrence of undetected reactions that suppress nitrite formation or reoxidize nitrite to nitrate. It may be envisaged that reactive species generated by decomposition of sulphonyl radicals (reaction 9) are responsible for such reactions. Indeed, this degradation process is greater for CSA than for HTAU and increases with increase in pH.

As reported in [33–35], *in vivo*, the reaction of peroxynitrite with CO_2 is particularly relevant because of its fast rate constant ($k = 2.6\text{--}3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7.4 and 25 °C) [33] and the ubiquity of the $\text{HCO}_3^-/\text{CO}_2$ pair in biological environments. Since the reaction of sulphinates with peroxynitrite is too slow (Figure 4) to outcompete CO_2 for the oxidant, it follows that, in the presence of added bicarbonate, the direct reaction of sulphinates with peroxynitrite would be inhibited and the oxidative reaction should involve only the radicals, $\bullet\text{NO}_2$ and $\text{CO}_3^{\bullet-}$, generated by homolysis of the short-lived peroxynitrite- CO_2 adduct [63–65]. As a consequence of these radical reactions, the sulphinic groups of HTAU and CSA are oxidized to the respective sulphonyl radicals ($\text{RSO}_2^\bullet$) that ultimately lead to the production of sulphonates through the oxygen-dependent reactions shown above. Accordingly, the results reported in Figure 6 show that, in the presence of added bicarbonate, the oxidation of sulphinates (1 mM) by peroxynitrite (200 μM) decreases, but the observed stoichiometry, i.e. mol of O_2 consumed/mol of depleted sulphinate, is similar to that obtained in the absence of bicarbonate. As discussed above, at this RSO_2^- concentration, sulphinates are oxidized mainly by a free radical mechanism even in the absence of bicarbonate. However, the peroxynitrite-derived radicals are different in the absence ($\bullet\text{OH}$ and $\bullet\text{NO}_2$) and presence of bicarbonate ($\text{CO}_3^{\bullet-}$ and $\bullet\text{NO}_2$). Unlike $\bullet\text{OH}$, which reacts with target molecules with rate constants close to the diffusion limit, $\text{CO}_3^{\bullet-}$ is a more selective radical that reacts with various biomolecules $10^2\text{--}10^7$ slower than $\bullet\text{OH}$ [40,66]. Thus it is possible that the lower extent of HTAU and CSA oxidation by peroxynitrite in the presence of bicarbonate may reflect, in addition to the lack of direct reaction, the lower reactivity of the carbonate radical towards sulphinates.

In conclusion, the above results indicate that peroxynitrite and its derived species can be included in the group of non-specific biological oxidants capable of accomplishing the oxidation of sulphinic groups of HTAU and CSA to sulphonic groups of TAU and CA respectively. However, the formation of intermediate sulphonyl radicals that can propagate oxidative reactions raises questions about the metabolic fate of these species [67]. Sulphonyl radicals are potent initiators of lipid peroxidation [68], behaving as oxidants that can exert damaging effects *in vivo*. Although our previous studies showed that HTAU and CSA can inhibit peroxynitrite-dependent reactions [46], the formation of reactive intermediates in the course of the oxidative reaction could have a pathophysiological significance that remains to be investigated.

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