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Original Contribution

Kinetics of reaction of peroxynitrite with selenium- and sulfur-containing compounds: Absolute rate constants and assessment of biological significance

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ABSTRACT

Peroxynitrite (the physiological mixture of ONOOH and its anion, $ONOO^-$) is a powerful biologically-relevant oxidant capable of oxidizing and damaging a range of important targets including sulfides, thiols, lipids, proteins, carbohydrates and nucleic acids. Excessive production of peroxynitrite is associated with several human pathologies including cardiovascular disease, ischemic-reperfusion injury, circulatory shock, inflammation and neurodegeneration. This study demonstrates that low-molecular-mass selenols (RSeH), selenides (RSeR') and to a lesser extent diselenides (RSeSeR') react with peroxynitrite with high rate constants. Low molecular mass selenols react particularly rapidly with peroxynitrite, with second order rate constants k_2 in the range 5.1×10^5–1.9×10^6 M^{-1} s^{-1}, and 250–830 fold faster than the corresponding thiols (RSH) and many other endogenous biological targets. Reactions of peroxynitrite with selenides, including selenosugars are approximately 15-fold faster than their sulfur homologs with k_2 approximately 2.5×10^3 M^{-1} s^{-1}. The rate constants for diselenides and sulfides were slower with k_2 0.72–1.3×10^3 M^{-1} s^{-1} and approximately 2.1×10^2 M^{-1} s^{-1} respectively. These studies demonstrate that both endogenous and exogenous selenium-containing compounds may modulate peroxynitrite-mediated damage at sites of acute and chronic inflammation, with this being of particular relevance at extracellular sites where the thiol pool is limited.

1. Introduction

Peroxynitrous acid (ONOOH) is a potent oxidant and nitrating species that is formed in vivo and in vitro by the cross-reaction (termination) of two biologically-important radicals: superoxide ($O_2^{\bullet -}$; generated by a respiratory burst from activated leukocytes) and nitric oxide ($NO^{\bullet}$; generated by nitric oxide synthase enzymes present in multiple cells) [1–3]. This species, and to a lesser extent its anion $ONOO^-$ (pK_a ~6.8, though this is buffer dependent [4,5]), oxidizes a wide variety of biomolecules including thioethers (such as methionine [6]), thiols [7], lipids [8,9], proteins (e.g. [10,11]), carbohydrates [12] and nucleic acids [13–15] either through direct two-electron oxidation, or via limited generation of reactive secondary radicals such as $HO^{\bullet}$ and $NO_2^{\bullet}$ [16–18]. In the presence of bicarbonate (HCO_3^-) additional reactions involving the peroxynitrosocarbonate anion ($ONOOCO_2^-$), and radicals derived from homolysis of this species ($HO^{\bullet}$ and $CO_3^{\bullet -}$) also play a role in inducing oxidative damage [17].

Excessive, mistimed or misplaced production of peroxynitrite (the physiological mixture of ONOOH and $ONOO^-$) has been associated with the development of organ damage and dysfunction in a number of pathologies including cardiovascular disease [19–21], ischemia-reperfusion injury [22,23], liver damage [24], circulatory shock [23], central nervous system damage [25], and neurodegeneration [26,27], amongst others. In such circumstances endogenous cellular and extracellular defense mechanisms may be overwhelmed, and synthetic materials that react rapidly with peroxynitrite may represent novel pharmacological protective agents for such conditions [28]. A number of low-molecular-mass compounds have been postulated as biological targets for, or protective agents against, peroxynitrite-mediated damage

Abbreviations: GSH, glutathione; HOX, hypohalous acids; MPO, myeloperoxidase; Sec, selenocysteine; SeMet, selenomethionine

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including thiols (e.g. glutathione, GSH), ascorbate, carbon dioxide (CO_2) and synthetic metalloporphyrins [29–32]. Additional studies have shown that reaction of peroxynitrite with some proteins including peroxidases, peroxiredoxins, hemoglobin, albumin and some selenoproteins is very rapid [29–34]. In the light of the concentrations of these species present in vivo it has been postulated that reaction with CO_2 (to give the nitrosoperoxycarbonate anion [35,36]) and GSH is likely to be quantitatively the most significant [32], though kinetic data for the reactions of peroxynitrite particularly with seleno-species are incomplete.

Selenium, in the form of selenocysteine (Sec), a RNA coded amino acid, plays a critical role in the activity of a number of cellular protective enzymes including glutathione peroxidase (GPx), thioredoxin reductase (TrxR), and some isoforms of methionine sulfoxide reductase [37,38]. GPx in conjunction with its co-factor, glutathione (GSH), catalyzes the reduction of H_2O_2 (and for GPx4, lipid hydroperoxides [39]) and peroxynitrite [40]. The reactions of GPx and peroxiredoxins (and related species) with peroxynitrite have been postulated to occur via protein-bound Sec and Cys residues respectively, with second order rate constants (k_2) in the range 10^5–10^7 M^{-1} s^{-1} (e.g. [30,33,34,41–44]). However it is unclear whether the high reactivity of these residues, and particularly Sec, is an inherent property of the free amino acid (and related seleno compounds), or a function of the surrounding protein. The synthetic selenoamide ebselen, and the natural amino acid selenomethionine (SeMet) are known to react more rapidly with peroxynitrite than their sulfur analogs, though k_2 for these low-molecular-mass species are, at least for SeMet, considerably slower ($\sim 1.5 \times 10^3$ M^{-1} s^{-1} [45]) than for the Sec residue of GPx. We have recently reported novel selenium-containing carbohydrates that react with HOX (X=Cl, Br, SCN) with high rate constants, and protect both isolated and plasma proteins from hypochlorous acid (HOCl)- and chloramine-mediated damage [46–48] (reviewed [49]). In the light of the high reactivity of these seleno compounds with HOCl we hypothesized that low-molecular-mass selenols, seleno sugars, and diselenides would also react rapidly with peroxynitrite. The absolute rate constants reported here for these reactions support this hypothesis. These rate constants and those for their sulfur analogs obtained for reaction with peroxynitrite, are also compared to other oxidants to allow the biological significance of these reactions and the role of structure in determining the reaction kinetics to be assessed.

2. Materials and methods

2.1. Materials

3,3′-Diselenodipropionic acid was synthesized and purified as described previously [50]. The selenosugars were synthesized as described previously [46,47]. The diselenide of selenocysteine methyl ester [-$SeCH_2CH(NH_2)C(=O)OCH_3]_2$ was synthesized as reported previously [51]. All other chemicals were from Sigma-Aldrich/Fluka and used without further purification. All selenium-containing compounds were dissolved in Chelex-treated sodium phosphate buffer (pH 7.4, 200 mM) prepared using Milli Q water. Peroxynitrite was prepared as described previously and stored in 0.1 M NaOH (pH ca. 12) [52]. Concentrations of the stock peroxynitrite solutions were determined prior to use by spectrophotometric quantification of its anion form ($ONOO^-$) at high pH using a previously reported extinction coefficient [53]. Immediately before use, peroxynitrite was diluted with MilliQ water to give 200 μM $ONOO^-$ in 5 mM NaOH. The strong buffering capacity (200 mM phosphate buffer) of the solutions containing the selenium compounds prevented changes in pH on mixing (1:1, resulting in a final buffer concentration of 100 mM) with the alkaline $ONOO^-$ stock solutions.

2.2. Kinetic measurements

Stopped-flow studies were carried out using an Applied Photophysics SX.18 MV stopped-flow system as described previously [54,55]. Each stopped-flow measurement was repeated 10 times with the data averaged to improve the signal to noise ratio.

Rate constants for the reactions of peroxynitrite with the selenol (reduced, RSeH) forms of Sec, selenocystamine, 3-selenopropionic acid [IUPAC name: 3-(hydroseleno)propanoic acid], and selenocysteine methyl ester, were determined by direct kinetics. The selenols were prepared in situ by reduction of the corresponding diselenide with a 10-fold excess of $NaBH_4$ under an argon atmosphere, as described previously [55]. The reduced selenols were then diluted with deoxygenated phosphate buffer (200 mM) to the required concentrations, before reaction with peroxynitrite (100 μM) at 22 °C under a N_2 atmosphere, with absorption changes determined at 245 nm (Sec, selenocystamine, and selenocysteine methyl ester) or 248 nm (3-selenopropionic acid). At these wavelengths peroxynitrite does not absorb to a significant extent, thus the selenol concentration for each kinetic run (50–385 μM) was determined by extrapolating the data to obtain the initial absorbance value ($t=0$ s) and converting to concentration using the corresponding molar absorption coefficients [55]). In the absence of peroxynitrite the selenols undergo slow autoxidation under the conditions employed [55], thus in alternative determinations of these rate constants, the absorption spectrum of the stock solutions of the selenol was measured before and after use, with the mean value of the two measurements used to estimate the selenol concentration.

As the kinetic traces obtained for the reaction of selenols with peroxynitrite were not always obtained under pseudo first-order conditions (i.e. with substrate excesses greater than 5-fold over oxidant), the decay profiles were analyzed using second-order decay profiles using Pro-KIV (Version 1.0.4.0; Applied Photophysics). In order to fit these traces a simple model was used comprising of two reactions: (i) RSeH + peroxynitrite → product, k_{RSeH} and (ii) peroxynitrite → decay products, k_{decomp}. The initial reactant concentrations were defined (following determination as described above) and the value of k_{decomp} was fixed as 0.27 s^{-1}, as determined from experiments with peroxynitrite in the absence of selenols (see Supplementary Fig. 1a). This value of k_{decomp} gives a rate constant for isomerization of peroxynitrite ($k_{isomerization}$) of ~ 1.3 s^{-1} at pH 7.4 and 22 °C in 100 mM phosphate buffer (where the pKa of peroxynitrite is ~6.8 [4,56]) using the equation reported by Molina et al. [57]. This value is slightly higher than that reported previously (1.1 s^{-1}) for highly purified peroxynitrite solutions [57]. In order to further reduce the number of fitting parameters, the peroxynitrite and peroxynitrite decay products were defined as non-absorbing species at the wavelengths employed. The model was then allowed to vary the value of k_{RSeH} to obtain the best fit to the data (Supplementary Fig. 1b and c shows example data and fits), which was typically achieved within 10–15 iterations. The k_{RSeH} values obtained for each kinetic trace ($n > 6$) were then averaged to obtain the k_2 values for each selenol, with errors reported as 95% confidence limits.

The reactions of peroxynitrite (100 μM) with selenides (RSeR', 0.5–1.25 mM), diselenides (RSeSeR', 0.5–1.25 mM), sulfides (RSR', 2.5–12.5 mM) and disulfides (RSSR', 2.5–12.5 mM) were determined by direct kinetic measurements of the loss of peroxynitrite measured at 302 nm. As higher concentrations were used for these substrates, the kinetic data could all be fitted to single exponential decays using Pro-Data viewer 4.0 (Applied Photophysics) and OriginPro 7.0 (OriginLab) software. For each substrate the resulting pseudo first order rate constant (k_{obs}) was plotted against

substrate concentration and the second order (bimolecular) rate constant k_2 was obtained from a linear fit of the data (with the intercept fixed as 0.27 s^{-1}, consistent with the natural decay of peroxynitrite measured in these studies). All rate constants reported for selenides, diselenides, sulfides and disulfides were derived from >3 independent experiments employing at least four different substrate concentrations and errors are specified as 95% confidence limits.

3. Results

3.1. Determination of rate constants for reaction of peroxynitrite with low-molecular-mass selenols

Selenols (RSeH) are structurally similar to thiols (RSH), but have lower pK_a values (i.e. are stronger acids) and exist primarily in their anion form (RSe^-, pK_a 5.2 for Sec) at neutral pH [58]. Selenols are

Table 1
Summary of rate constants, k_2, determined for reaction of peroxynitrite with seleno- and compounds using stopped-flow methods with absorbance detection. Reactions were carried out in 0.1 M phosphate buffer, pH 7.4 at 22 °C. Second-order rate constants are given with 95% confidence limits. Literature data for the corresponding sulfur-analogues are provided for comparison.

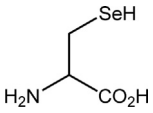
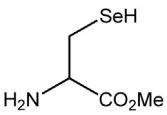
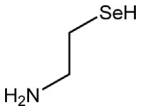
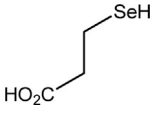
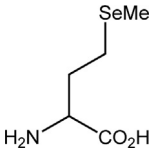
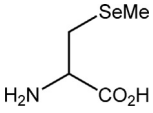
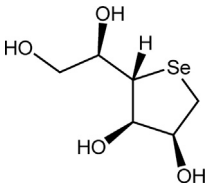
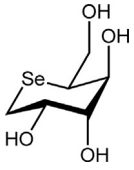
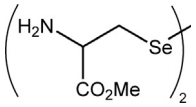
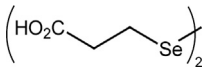
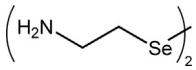
Substrate	Structure	k_2 / $M^{-1}s^{-1}$	k_2 / $M^{-1}s^{-1}$ (for sulfur analogue)
Selenocysteine (Sec)		$(6.6 \pm 0.3) \times 10^5$ [a]	$(2.6 \pm 0.1) \times 10^3$ [b]
Sec methyl ester		$(1.2 \pm 0.7) \times 10^6$ [a]	$(3.9 \pm 0.1) \times 10^3$ [b]
Selenocysteamine		$(1.9 \pm 1.0) \times 10^6$ [a] $(7.1 \pm 0.4) \times 10^5$ [c]	$(2.3 \pm 0.1) \times 10^3$ [b]
3-Selenopropionic acid		$(5.1 \pm 0.7) \times 10^5$ [d]	ND [e]
Selenomethionine (SeMet)		$(2.5 \pm 0.1) \times 10^3$ [b] 1.48×10^3 (at 25 °C [45])	$(1.6 \pm 0.1) \times 10^2$ [b] [$(1.7 \pm 0.1) \times 10^3$ for acid, and (8.6 ± 0.2) for anion [6]
(Se-methyl)-selenocysteine		$(1.9 \pm 0.1) \times 10^3$ [b]	ND [e]
Selenotalose (SeTal)		$(2.5 \pm 0.1) \times 10^3$ [b]	ND [e]
Selenogulose (SeGul)		$(2.5 \pm 0.1) \times 10^3$ [b]	$(2.1 \pm 0.1) \times 10^2$ [b]
Sec methyl ester diselenide		$(7.3 \pm 0.3) \times 10^2$ [b]	ND [e]

Table 1 (*continued*)

Substrate	Structure	k_2 / $M^{-1}s^{-1}$	k_2 / $M^{-1}s^{-1}$ (for sulfur analogue)
3,3′-Diseleno-dipropionic acid		$(1.3 \pm 0.1) \times 10^3$ [b]	ND [e]
2,2′-diseleno-cystamine		$(7.2 \pm 0.4) \times 10^2$ [b]	ND [e]

[a] Absorbance changes measured at 243 nm to monitor loss of selenol, with extinction coefficient used to calculate selenol concentration.
[b] Absorbance changes measured at 302 nm to monitor loss of $ONOO^-$.
[c] Value determined using absorption spectra of selenol before and after kinetic runs.
[d] Absorbance changes measured at 248 nm to monitor loss of selenol, with extinction coefficient used to calculate selenol concentration.
[e] ND, not determined.

powerful nucleophiles and undergo ready oxidation in air [58], so these materials were synthesized in their diselenide (RSeSeR') forms. The selenols were obtained from the diselenides for kinetic studies by in situ reduction with sodium borohydride [55]. The slow decay of the stock selenol solutions was accounted for using two different approaches (see Section 2), which gave k_2 values of similar magnitude. The rate constants for reaction with peroxynitrite were determined under an atmosphere of N_2 by direct stopped-flow methods using final concentrations of 50–385 μM selenol and 100 μM peroxynitrite at 22 °C over a period of 0–0.2 s, with changes in absorbance from the selenol monitored at its λ_{max} [55]. Control experiments with decomposed peroxynitrite in buffer confirmed that decay products from the oxidant had minimal impact on the selenol reaction data. The observed absorbance changes on mixing peroxynitrite and selenols were fitted directly to second-order kinetics (for example data and fits see Supplementary Fig. 1b and c) to obtain an estimate of the second-order rate constants (k_2) at each selenol concentration; these values were then averaged and the 95% confidence intervals were calculated (Table 1).

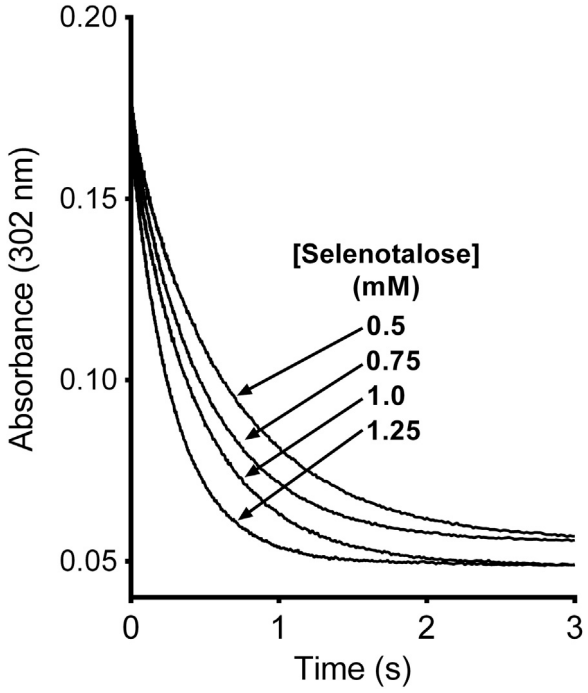


Fig. 1. Representative stopped-flow kinetic traces for the reaction of SeTal (0.5–1.25 mM) with peroxynitrite (0.1 mM) at 22 °C in 0.1 M phosphate buffer (pH 7.4). The kinetic data obtained at 302 nm are represented by averaging 10 experiments with the observed rate constant (k_{obs}) generated from the exponential fitted to a single continuous decay curve.

3.2. Determination of rate constants for reaction of peroxynitrite with low-molecular-mass selenides, diselenides and sulfur analogs

Unlike selenols that have a strong absorbance band in the UV region (< 320 nm), selenides (selenoethers, RSeR'), diselenides (RSeSeR'), thiols (RSH), thioethers (RSR') and disulfides (RSSR') do not have significant confounding UV–visible absorbance bands at the wavelengths examined. As a consequence the kinetics of these reactions were examined by quantifying the enhanced rate of decay of peroxynitrite at 302 nm in the presence of these chalcogen compounds. Selenoethers, thiols or diselenides (0.5–1.25 mM) were reacted with 100 μM peroxynitrite at 22 °C over a period of 0–5 s, with changes in absorbance measured at 302 nm. Thioethers or disulfides (2.5–12.5 mM) were reacted with 100 μM peroxynitrite in a similar manner but over a period of 0–8 s. Control experiments in the absence of substrate showed that the spontaneous decay of peroxynitrite at pH 7.4 was less rapid (Supplementary Fig. 1a; k 0.27 s^{-1}), and not a confounding reaction over these timescales. Absorbance changes were fitted to a single-exponential function to give k_{obs} (Fig. 1) with subsequent

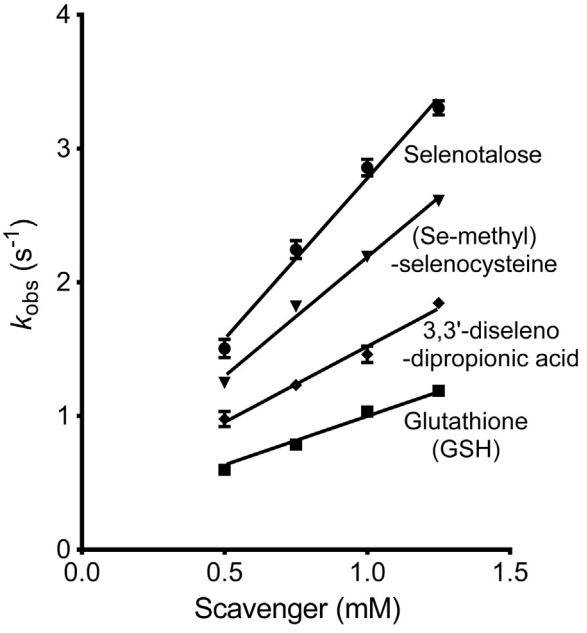


Fig. 2. Plots of observed rate constants (k_{obs}) against scavenger concentrations for a range of selenides, diselenides and glutathione (GSH). The second order rate constants, k_2, for reaction with peroxynitrite were established from the gradients of these linear plots. Error bars represent ± SEM (n=3). The y-intercept was fixed as 0.27 s^{-1}, the experimentally-determined rate constant for decay of peroxynitrite in the absence of added compounds (see text and Supplementary Fig. 1).

plots of k_{obs} against substrate concentration, giving a straight line (Fig. 2) from which k_2 was obtained from the gradient (Table 1). Kinetic data for 3,3′-diselenocystine could not be acquired due to its low solubility under the conditions employed and the requirement for high substrate excesses.

4. Discussion

Peroxynitrite is a major oxidant generated at sites of inflammation that is both bactericidal and potentially damaging to host tissues (reviewed: [16,17,59]). Peroxynitrite can react directly and rapidly, with proteins, metal ions, selenium-containing proteins and pharmaceuticals, and (via its anion form $ONOO^-$) with CO_2 to give the peroxynitrosocarbonate anion ($ONOOCO_2^-$) [11,17,59,60]. Homolytic cleavage of both ONOOH and $ONOOCO_2^-$ can result in radical formation and additional damage [17,59,61], though this homolysis reaction occurs to only a very limited extent, and its quantitative significance is unclear [18].

The reactivity of peroxynitrite with common amino acids and the Sec residue of GPx has been examined previously [11,30]. Cys, Met and Trp are the only free amino acids that undergo appreciable direct reaction with peroxynitrite, but other amino acids (e.g. Tyr, His and Lys) can be modified via secondary radicals, or species generated in the presence of transition metal ions [10,59]. The rate constants for reaction of peroxynitrite with the Sec residue of GPx, and Cys residues of peroxiredoxins, are some of the highest known for peroxynitrite with k_2 in the range $\sim 10^5$– 7×10^7 M^{-1} s^{-1}, with peroxiredoxins reported to be major targets for this oxidant [30,41,43,44,62]. In contrast, k_2 values for reaction with low-molecular-mass thiols (Cys, GSH) or SeMet have been reported to be in the range $1.4–2.0 \times 10^4$ M^{-1} s^{-1} [7,63,64]. Reaction with thioethers such as Met has $k_2 \sim 1.7 \times 10^3$ M^{-1} s^{-1} [6]). In the light of these, and previous data [55], it was hypothesized that reaction of peroxynitrite with selenols should be significantly more rapid than with analogous thiols, selenides and sulfides, as selenols are predominantly ionized at neutral pH values (cf. pK_a's of 5.2 and 8.4 for Sec and Cys respectively [58,65] and are more nucleophilic than sulfur centers. However it should be noted that the reactivity of these compounds is unlikely to be determined solely by pK_a values. Thus it has been reported that changing the pK_a value of a Cys residue from 8.4 to 5 only changes the reactivity by a maximum of 10-fold at pH 7.4 as the thiolate availability increases from ~10% to 100% [66], and many proteins with low pK_a Cys residues react slowly with peroxides or peroxynitrite. The situation with glutathione peroxidases (GPxs) and peroxiredoxins (Prxs) is however different, as these are specialized peroxidases that have evolved to maximize peroxide and peroxynitrite reduction [33].

The data presented here are consistent with much greater reactivity of the selenium-containing species compared to their sulfur analogs. The values of k_2 for reaction of peroxynitrite with selenols are between 250 and 830-fold higher than for the corresponding thiols, and up to 2600-fold higher than for the corresponding (oxidized) diselenides under identical conditions (Table 1). Selenols also react approximately 500-fold faster than selenides (selenoethers, e.g. SeMet), and up to 5500-fold faster than sulfides (thioethers, e.g. Met). The value for k_2 determined here for free Sec is lower than that for the active site Sec of GPx (Table 2), consistent with the hypothesis that the protein structure

Table 2
Comparison of second order rate constants, k_2, for selected reactions of biologically-relevant oxidants with seleno- and sulfur- compounds at pH 7.4 and ~22 °C unless indicated otherwise. Data are from the current study unless otherwise noted. For information on the associated errors see Table 1 and cited literature.

Substrate	k (HOCl) (M^{-1} s^{-1})	k (HOSCN) (M^{-1} s^{-1})	k (ONOOH) (M^{-1} s^{-1})
Cysteine	3.1×10^8 [84]	7.8×10^4 [54]	2.6×10^3 4.5×10^3 (at 37 °C [7]) For Arrhenius plot and further data [5,85]
Selenocysteine	ND	1.2×10^6 [55]	6.6×10^5 [a]
N-Ac-cysteine	2.9×10^7 [84]	7.3×10^3 [54]	4.15×10^2 (at 37 °C [63])
Cystamine	ND	7.8×10^4 [54]	2.3×10^3
Selenocystamine	ND	5.8×10^6 [55]	1.9×10^6 [a] 7.1×10^5 [b]
GSH	1.1×10^8 [84]	2.5×10^4 [54] 8.0×10^4 (at 25 °C [86])	7.3×10^2 1.36×10^3 (at 37 °C [5,63])
Methionine	3.4×10^7 [84]	$< 10^3$ [54]	1.6×10^2 1.7×10^3 for ONOOH, and 8.6 for $ONOO^-$ [6] 3.64×10^2 (at 37 °C [87])
Selenomethionine	3.2×10^8 [47]	2.8×10^3 [55]	2.5×10^3 1.48×10^3 (at 25 °C [45])
Selenotalose	1.0×10^8 [47]	ND	2.5×10^3
Selenogulose	9.4×10^7 [47]	ND	2.5×10^3
Ebselen	ND	3×10^1 [55]	2×10^6 (at 25 °C, pH $\geq$ 8) [88]
Serum albumin	ND	7.6×10^4 (for bovine serum albumin [54])	9.7×10^3 (for human serum albumin at 37 °C [87]) 3.8×10^3 (for Cys residue of human serum albumin at 37 °C [87])
Glutathione peroxidase	ND	5×10^5 [55]	8×10^6 [30] 1.8×10^5 (at 37 °C, pH 7.1 [89]) 7.4×10^5 (for oxidized tetramer; [30]) $\sim 10^6$ (for poplar thioredoxin-dependent glutathione peroxidase 5 [90])
Peroxiredoxin family	ND	[c]	7×10^7 for human peroxiredoxin 5 [41,62] 1.5×10^6 for AhpC (from *Salmonella typhimurium* [43]) 3.0×10^3 (for oxidized AhpC [43]) $\sim 10^5$ for thioredoxin peroxidases I/II (from *Saccharomyces cerevisiae* [44])

ND: not determined.

[a] Value determined using extinction coefficient to calculate selenol concentration.
[b] Value determined using absorption spectra of selenol before and after kinetic runs.
[c] Data for peroxiredoxins not determined; for other Cys-containing proteins k_2 is $1.0–7.6 \times 10^4$ M^{-1} s^{-1} [54].

modulates the environment and reactivity of the selenol. This conclusion is supported by the variation in k_2 between the various selenols (Table 1), with the data being consistent with the neighboring groups modulating selenol reactivity. Of the selenols examined, the lowest k_2 values were for 3-selenopropionic acid and Sec (both approximately $5 \times 10^5\ M^{-1}\ s^{-1}$) with higher values determined for Sec methyl ester (k_2 $1.2 \times 10^6\ M^{-1}\ s^{-1}$) and reduced selenocystamine (k_2 $7.1–19.0 \times 10^5\ M^{-1}\ s^{-1}$). A similar trend is apparent for the corresponding thiols, though these values were much lower than for the selenium analogs, with the highest values determined for the methyl ester of cysteine ($3.9 \times 10^3\ M^{-1}\ s^{-1}$). These data suggest that the presence of a suitably positioned ionized carboxyl group decreases the rate of reaction, and a protonated amine group enhances the rate of reaction, though the exact reason for this observation remains to be established.

Previous studies have reported k_2 for reaction of peroxynitrite with Cys to be $4.5 \times 10^3\ M^{-1}\ s^{-1}$ [7] and $1.36 \times 10^3\ M^{-1}\ s^{-1}$ at 37 °C for GSH [63]. These values are between 1.7 and 1.9-fold faster than determined here at 22 °C. The similarity of the ratio of the k_2 values at these two different temperatures for Cys and GSH, suggests that realistic estimates can be made for other species at 37 °C, if required.

All the selenides examined had similar k_2 values, with both the novel 6-membered ring seleno-sugar SeGul, and the 5-membered ring species SeTal, being $2.5 \times 10^3\ M^{-1}\ s^{-1}$. Interestingly 3-(selenomethyl)cysteine ($1.9 \times 10^3\ M^{-1}\ s^{-1}$) displayed a slightly lower rate constant than SeMet ($2.4 \times 10^3\ M^{-1}\ s^{-1}$), suggesting that an increased distance between the seleno center and the other functional groups enhances reactivity; this may reflect a diminution of through bond electron withdrawing effects. Comparison of these data with those of the corresponding sulfur-analogs (e.g. thiogulose, SGul, k_2 $2.1 \times 10^2\ M^{-1}\ s^{-1}$) indicates an approximate 12-fold decrease in reactivity, consistent with the difference between SeMet and Met (Table 1).

For the diselenides the highest values of k_2 were determined for 3,3′-diselenodipropionoic acid ($1.3 \times 10^3\ M^{-1}\ s^{-1}$) with lower values determined for the 3,3′-diselenide form of the Sec methyl ester ($7.3 \times 10^2\ M^{-1}\ s^{-1}$) and 3,3′-diselenocystamine ($7.2 \times 10^2\ M^{-1}\ s^{-1}$). These values indicate that diselenides per se offer limited scavenging activity against peroxynitrite in biological systems when compared to their reduced (selenol) forms. However as diselenides are likely to be reduced in vivo (cf. the ready reduction of disulfides to thiols), these species may act as potential pro-forms of the more reactive selenols. Data for the analogous disulfide reaction could not be obtained due to the (relatively) rapid intrinsic decay of peroxynitrite under these conditions, consistent with a previous report for glutathione disulfide where no increased rate of peroxynitrite decay was observed [63]. The cyclic disulfide lipoic acid does however enhance the rate of peroxynitrite decay (k_2 $1.4 \times 10^3\ M^{-1}\ s^{-1}$ at 37 °C), with this resulting in disulfide S-oxide (thiosulfinate) formation [63].

Although the present study has provided absolute kinetic data for the reactivity of peroxynitrite with selenols and other selenospecies, the mechanisms and products of these reactions remain to be fully defined. The major pathway for the analogous thiol reactions has been reported to be a (2-electron) molecular process involving nucleophilic attack of the thiolate anion on one of the peroxidic oxygens of peroxynitrite resulting in the formation of an intermediate sulfenic acid (RSOH), which then reacts rapidly with another thiol to give the disulfide [7]. The RSOH intermediate has been detected in some cases (e.g. on peroxiredoxins [43] and albumin [67]). However radicals that may be generated as a result of peroxynitrite homolysis (either in the absence or presence of CO_2) may also mediate thiol oxidation via formation of thiyl ($RS^{\bullet}$), disulfide radical-anion ($RSSR^{\bullet -}$) and sulfinyl ($RSO^{\bullet}$) radicals, with these and other species participating in dioxygen-dependent chain reactions. These reactions also generate nitroso, nitro, nitroso-thiol and nitro-thiol products, in addition to the disulfides [68–72]. The identities of the materials formed from the seleno species remain to be determined, though it is probable that the major route is a 2-electron (molecular) reaction to give a selenenic (RSeOH) species before reaction with another selenol, to give the diselenide.

The data reported in Table 1 allow conclusions to be drawn as to the potential importance of peroxynitrite reactions with sulfur- versus seleno-species within cells and extracellular fluids. Within cells, the high concentration of low-molecular-mass thiols (e.g. GSH; 2–5 mM [73]) compared to sub micromolar levels of *endogenous* low-molecular-mass selenols/selenides/diselenides, suggests that there will be a marked selectivity for thiol oxidation, despite the higher reactivity of selenols/selenides/diselenides. In the case of exogenously added species (supplementation), high μM levels of added seleno compounds would be needed before direct seleno oxidation becomes significant.

With Sec residues on proteins, the situation is more favorable. As thioredoxin reductase is only present at nM levels in cells [74] this is unlikely to be a major target when compared to GSH or protein thiols. In contrast, cellular concentrations of GPx are high (~2 μM [74,75]) and may compete with GSH for peroxynitrite in the light of the values presented in Table 2. These data are consistent with a report that GPx can act as a peroxynitrite reductase, thereby preventing peroxynitrite-induced damage [76]. A similar argument applies to intracellular protein-bound Cys residues, which are present at millimolar concentrations (cf. protein thiol concentrations in mitochondria of 60–90 mM [77,78]), though the higher k_2 values for some protein-Cys residues compared to GSH (cf. Table 2), may mitigate against this. In particular, proteins with low pK_a Cys residues (e.g. cytosolic peroxiredoxins, Prx1 and Prx2, which are present in Jurkat cells at ~65 μM, and mitochondrial Prx3 which is present at ~125 μM [79]) would be expected to outcompete reaction with GPx.

In extracellular fluids, the situation is very different, as the concentration of the low-molecular-mass thiol pool in plasma is low (cf. 12–19 μM) [80], compared to the concentration of protein-incorporated Cys (primarily Cys-34 of human serum albumin; nominally 400–600 μM, although a significant fraction of this is present in modified forms [80]). At these thiol concentrations, and with a 250–830 greater reactivity of selenols over thiols with peroxynitrite (Table 1), reaction of peroxynitrite with low-molecular-mass plasma selenols, selenides and diselenides would be competitive if these were present at low μM levels. For extracellular selenoproteins, the (up to) 10 Sec residues of selenoprotein P (the most abundant seleno species in plasma [81]) would be expected to be competitive for peroxynitrite (relative to both free- and protein Cys residues) on the basis of the determined k_2 value for free Sec (Tables 1 and 2), and the reported plasma concentrations of this seleno protein, which range from 0.8 to 2 μM, and hence a total Sec concentration of up to 20 μM [81]. This conclusion is consistent with experimental data [29]. Plasma concentrations of extracellular GPx (GPx3) and thioredoxin reductase are lower: 0.24–0.6 μM (and hence 0.96–2.4 μM Sec given its tetrameric structure) and 0.3 nM respectively [74, 82]. On the basis of these data, reaction of peroxynitrite with the Sec residues of GPx3 may be significant (given a concentration ratio of protein Sec to Cys of 0.96–2.4 μM : 400–600 μM, and a k_2 ratio of ~800; cf. Table 2), but scavenging of peroxynitrite by extracellular thioredoxin reductase would not.

Overall, these data indicate that selenols and other selenospecies are targets for peroxynitrite, with rate constants for selenols being significantly faster than for thiols, and selenides being faster than sulfides/thioethers. Diselenides also exhibit considerable reactivity towards peroxynitrite, though their k_2 values are lower than for both selenols and selenides. The diselenides are

however more reactive than most disulfides, which react very slowly with peroxynitrite [11,63].

The data in Table 2 indicate that peroxynitrite is considerably less reactive than a number of other inflammatory oxidants including HOCl and hypothiocyanous acid (HOSCN), with these targets. Thus HOCl reacts with thiols approximately five orders of magnitude faster than peroxynitrite ($\sim 10^8$ versus $\sim 10^3\ M^{-1}\ s^{-1}$; Table 2), and a similar enhancement is seen for thioether : selenide pairs (e.g. Met vs. SeMet). This difference diminishes for proteins, with the k_2 values for peroxynitrite being closer to those of other oxidants for both highly-reactive protein thiols, and protein-bound Sec residues (cf. data for GPx and peroxiredoxins in Table 2) although comparison with the reactions of HOCl is not possible due to the rapidity of these reactions. The differences in rate constants between peroxynitrite and HOSCN, an oxidant generated by multiple heme peroxidase enzymes such as myeloperoxidase, eosinophil-, lacto- and gastric-peroxidases [83]), are less dramatic but the data for HOSCN suggest it is more reactive than peroxynitrite with thiols and selenols, with the k_2 values being 2 to 5-fold higher. Overall, these data are consistent with the oxidants generated by both neutrophil- and macrophage-derived processes being important in both pathogen removal, and host tissue damage, with these mechanisms being both complementary and redundant (i.e. capable of replacing each other, at least to a major extent).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.10.424.

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