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Selenium Redox Cycling in the Protective Effects of Organoselenides against Oxidant-Induced DNA Damage

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Abstract: The biological role of selenium is a subject of intense current interest, and the antioxidant activity of selenoenzymes is now known to be dependent upon redox cycling of selenium within their active sites. Exogenously supplied or metabolically generated organoselenium compounds, capable of propagating a selenium redox cycle, might therefore supplement natural cellular defenses against the oxidizing agents generated during metabolism. We now report evidence that selenium redox cycling can enhance the protective effects of organoselenium compounds against oxidant-induced DNA damage. Phenylaminoethyl selenides were found to protect plasmid DNA from peroxynitrite-mediated damage by scavenging this powerful cellular oxidant and forming phenylaminoethyl selenoxides as the sole selenium-containing products. The redox properties of these organoselenoxide compounds were investigated, and the first redox potentials of selenoxides in the literature are reported here. Rate constants were determined for the reactions of the selenoxides with cellular reductants such as glutathione (GSH). These kinetic data were then used in a MatLab simulation, which showed the feasibility of selenium redox cycling by GSH in the presence of the cellular oxidant, peroxynitrite. Experiments were then carried out in which peroxynitrite-mediated plasmid DNA nick formation in the presence or absence of organoselenium compounds and GSH was monitored. The results demonstrate that GSH-mediated redox cycling of selenium enhances the protective effects of phenylaminoethyl selenides against peroxynitrite-induced DNA damage.

The biological role of selenium is attracting a great deal of current interest, and several selenium-containing proteins have been identified in mammalian tissues. Among these are the glutathione peroxidases (GPx) and thioredoxin reductase (TR), with the antioxidant activities of these selenoenzymes being critical to cellular survival.[1–6] Over the years, many researchers have proposed that the activity of selenoenzymes entails redox cycling of selenium within their active site.[6–8] Exogenously supplied or metabolically generated organoselenium compounds, capable of propagating a selenium redox cycle, might therefore supplement natural cellular defenses against the oxidizing agents, which are generated during normal metabolism and in disease states.

One of the most predominant cellular oxidants is peroxynitrite (ONOO⁻), a powerful oxidizing and nitrating agent formed from a diffusion-controlled reaction between nitric oxide (•NO) and superoxide $(O_2^{\bullet-})^9$ in endothelial cells, macrophages, and neutrophils.[10] Peroxynitrite reacts with various biological molecules, such as lipids, proteins, thiols, and metalloenzymes,[10,11] and it also reacts readily with DNA[12] where it causes base modification and induction of double- and single-strand breaks. Evidence indicates that reactions of peroxynitrite with cellular components are linked to oxidative stress and inflammatory tissue damage, as, for example, in atherosclerosis and ischemia–reperfusion injury.[13,14]

We now report evidence that selenium redox cycling—whereby the selenoxide produced from an organoselenide by peroxynitrite is then recycled back to the selenide by a cellular reductant—can enhance the protective effects of organoselenium compounds against peroxynitrite-induced DNA damage. We also report the first redox potentials of selenoxides ever reported in the literature. In the experiments described here, peroxynitrite-mediated plasmid DNA nick formation was monitored in the

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presence or absence of organoselenium compounds and gluta-thione (GSH)-based selenoxide redox cycling. Taken together with kinetic studies on the reactions of cellular reductants with selenoxides, potentiometric titrations, and cyclic voltammetry measurements, and a MatLab simulation of selenide regeneration by GSH in the presence of peroxynitrite, our results demonstrate that redox cycling enhances the protective effects of phenyl-aminoethyl selenides against oxidant-induced DNA damage.

Experimental Section

Materials. (L)-Ascorbic acid and sodium dithionite were obtained from Aldrich Chemical Co. (Milwaukee, WI). Reduced glutathione (GSH), 2-(*N*-morpholino)ethane sulfonic acid (MES), diethylenetriamine-pentaacetic acid (DTPA), (D,L)-selenomethionine, (D,L)-methionine, ethidium bromide, bromophenol blue, and Ficol (type 400) were obtained from Sigma Chemical Co. (St. Louis, MO). pUC 19 plasmid was generously provided by Dr. Kathryn B. Grant (Georgia State University, Atlanta, GA). Phenylaminoethyl selenides[15,16] [phenyl-2-aminoethyl selenide (PAESe), (*S*)-4-hydroxy-α-methyl-phenyl-2-aminoethyl selenide (HOMePAESe)], phenylaminoethyl sulfides[17] [phenyl-2-aminoethyl sulfide (PAES), 4-hydroxy-α-methyl-phenyl-2-aminoethyl sulfide (HOMePAES)], and their corresponding selen-oxides[16,18] and sulfoxides[18] were synthesized as previously described. Peroxynitrite was synthesized by the autoxidation of 10 mM hydroxyl-amine in 0.5 M aqueous NaOH solution containing 100 μM DTPA, as previously described.[18-20] All other chemicals and solvents were purchased from standard commercial sources and were of the highest grade available. HPLC columns (C-8) were obtained from Alltech (Deerfield, IL).

Reduction of Selenoxides and PAESO by Glutathione (GSH) and (L)-Ascorbate. The second-order rate constants for the redox reaction between the selenoxides, PAESO and GSH or (L)-ascorbate (AscH$^-$) were determined spectrophotometrically under pseudo-first-order rate conditions using the HP 8453 diode-array spectrophotometer equipped with an HP 89090 temperature-control accessory. Selenoxide or PAESO (50 μM) was combined with GSH (350-600 μM) in 100 mM potassium phosphate buffer (pH 7.0) at 25 °C in a quartz cuvette, and the absorbance changes were monitored over approximately a 30-s time interval. Due to sufficient differences in molar extinction coefficients of reactants and products, concentrations of selenide/selenoxide or sulfide/sulfoxide could be determined using the multicomponent module of the Biochemical Analysis UV–visible ChemStation software. The pseudo-first-order rate constants obtained from the concentration versus time plots were used to determine the second-order rate constant of the reaction. Separation of reactants and products was accomplished on a C-8 column (UV detection at 236 nm) with a mobile phase of 80% H$_2$O, 20% MeOH, and 0.1% TFA, at a flow rate of 1.5 mL/min. Authentic samples of GSH, GSSG, selenide/selenoxide, and sulfide/sulfoxide were used to confirm the identity of the reaction constituents.

Similar methodology was employed in the case of (L)-ascorbate as the reducing agent, whereas 100 μM AscH$^-$ was reacted with selenoxide/sulfoxide (0.40-1.0 mM) in 100 mM MES buffer (pH 5.5) at 37 °C. Reverse-phase HPLC (UV detection at 233 nm) was once again employed to separate the reaction products between the selen-oxides, PAESO, and ascorbate.

Potentiometric Titrations. The cell reduction potentials (*E*) for the selenoxides were obtained by potentiometric titration with the reducing

titrant, sodium dithionite (sodium hydrosulfite). Measurements were performed in a two-electrode-cell system with a single compartment containing a platinum spiral wire indicator electrode and an aqueous saturated calomel electrode (SCE) connected to a millivolt meter (Fisher Scientific, Norcross, GA). Measurements were carried out at 25 °C under argon in 100 mM sodium acetate, pH 5.5, with an initial concentration of selenoxide of 1 mM. Sodium dithionite (10 mM) was titrated into the cell with a 30-min equilibration period between each additional aliquot. All reported potentials were corrected to potentials versus the normal hydrogen electrode (NHE).

Cyclic Voltammetry. Peak potentials E_{pc} for the selenides, selen-oxides, sulfides and sulfoxides were obtained by cyclic voltammetry using a BAS 100B Electrochemical Analyzer (Bioanalytical System, U.S.A.). Measurements were carried out at 25 °C under argon, in 100 mM sodium acetate buffer, pH 5.5, in a conventional three-electrode-cell system with a single compartment. The working electrode was a glassy carbon electrode, and pretreatment was required prior to each measurement (i.e. the surface of the electrode was polished with powdered alumina and rinsed thoroughly with distilled H$_2$O). A platinum spiral wire served as a counter electrode, and SCE acted as the reference electrode. A scan rate of 100 mV/s was used for all compounds, and peak potentials were corrected to potentials versus the NHE.

Protection of Plasmid DNA (pUC19) from Peroxynitrite-Medi-ated Damage in the Presence of Organoselenium and Organosulfur Compounds. Peroxynitrite (final concentration 500 μM) was added to a solution of pUC19 plasmid DNA (25 ng/μL) in 200 mM phosphate buffer, pH 6.8, in the presence or absence of various organoselenium and organosulfur compounds (500 μM); the total reaction volume was 20 μL. The mixture was gently vortexed to initiate the reaction at 25 °C. The final pH of the reaction mixture was measured to be 7.4 and accounts for the shift caused by peroxynitrite addition. Following a 3-min incubation, 4 μL of electrophoresis loading buffer (0.05% bromophenol blue, 15% Ficol) was added to each reaction mixture, and an aliquot (23 μL) was loaded onto a 1% agarose gel containing 0.5 μg/mL ethidium bromide. The running buffer used contained 40 mM Tris/20 mM sodium acetate/1 mM EDTA with ethidium bromide (0.5 μg/mL). Following electrophoresis, gels were scanned and quantified using a Molecular Dynamics FluorImager SI Gel Imaging System. The percent DNA damage was determined using the equation:

(open circular DNA)/
(open circular DNA + supercoiled DNA × 1.22) × 100%

Peroxynitrite causes 88 ± 4% of supercoiled DNA to become nicked in the absence of any protecting agents, and this level of damage was set to 100%. Data are expressed as the mean ± SD (*n* = 3–4).

Recycling of Selenide by GSH in the Presence of DNA. Peroxy-nitrite (final concentration 250 μM) was added to a solution of pUC19 plasmid DNA (25 ng/μL) in 200 mM phosphate buffer, pH 7.5, in the presence or absence of HOMePAESe, HOMePAESeO, and/or GSH (250 μM), the total reaction volume was 20 μL. The mixture was gently vortexed to initiate the reaction at 25 °C. The final pH of the reaction mixture was measured to be 8.0 and accounts for the shift caused by peroxynitrite addition. The electrophoresis and gel quantitation proce-dures were carried out as described above. Peroxynitrite causes 54 ± 2.5% of supercoiled DNA to become nicked in the absence of any protecting agents, and this level of damage was set to 100%. Data are expressed as the mean ± SD (*n* = 6).

HPLC Analysis of pUC 19 Plasmid Assays. Reverse-phase HPLC (UV detection at 236 nm) was used to separate the reactants and products of peroxynitrite-mediated strand breakage. Diluted aliquots of reaction mixtures (20 μL) were injected onto a reverse phase C-8 column with a mobile phase of 15% acetonitrile, 85% water, and 0.1% trifluoroacetic acid (TFA), at a flow rate of 1.5 mL/min. Peroxynitrite,

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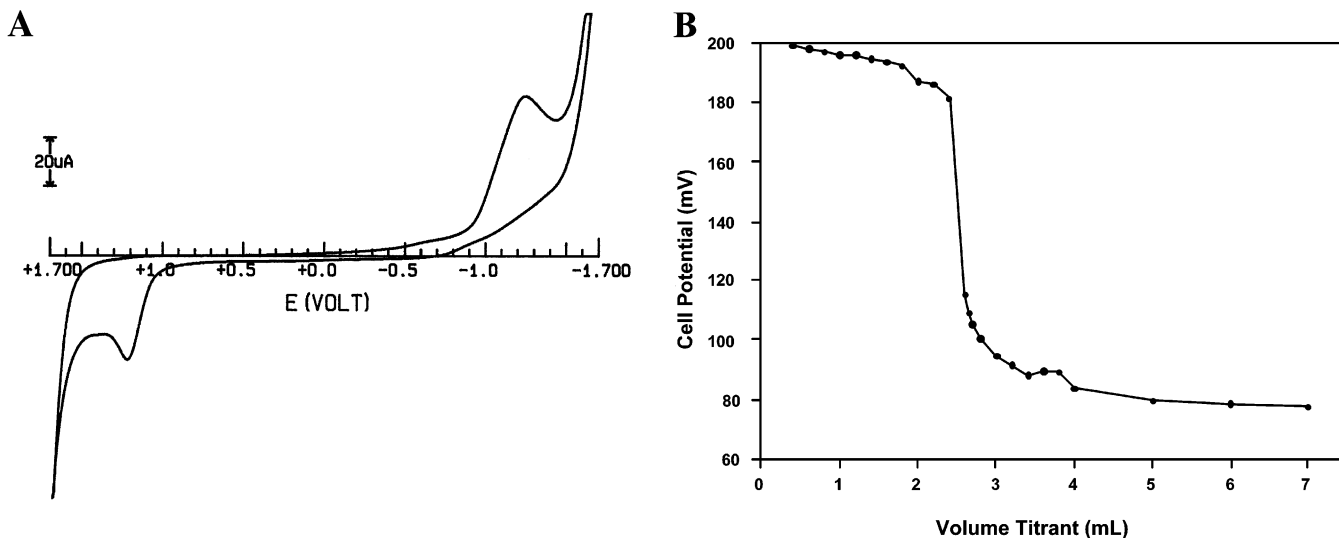
Figure 1. A typical potentiometric titration curve and cyclic voltammogram for 1 mM PAESeO. (A) Cyclic voltammetry experiments were performed in 100 mM sodium acetate, pH 5.5, in a conventional three-electrode cell as described in the Experimental Section. The initial potential was +1700 mV, the high potential was +1700 mV, the low potential was −1700 mV, and the scan rate was 100 mV/s. (B) Potentiometric titration experiments were carried out in 100 mM sodium acetate, pH 5.5, with 10 mM sodium dithionite as the titrant as described in the Experimental Section.

its reaction products, the products of pUC19 plasmid DNA strand breakage, and GSH/GSSG eluted in the void while HOMePAESeO and HOMePAESe eluted at approximately 3 and 7 min, respectively.

Results and Discussion

Enzymatic selenoxidation of phenylaminoethyl selenides has been extensively investigated both in vitro and in chromaffin granule ghosts.[15,16,21] Moreover, phenylaminoethyl selenoxides, but not the corresponding sulfoxides, have been shown to consume reduced ascorbate within the chromaffin granule,[16] and it has been proposed that this is a reflection of redox cycling of the selenium moiety of these compounds within the chromaffin granule. However, we were surprised to find that redox potential data for selenoxides have not been heretofore reported in the literature.

Potentiometric titrations of phenylaminoethyl selenoxides were performed in aqueous solution, pH 5.5, using the reducing agent, sodium dithionite (Figure 1A). The redox potentials, E, ranged from +410 to +480 mV (Table 1), with electron withdrawing p-substitution resulting in an increased potential. Cyclic voltammetry experiments were also carried out in aqueous solution, pH 5.5, and all selenoxides were found to be electro-active within a reasonable potential range; however, they exhibited irreversible or quasi-reversible behavior as evidenced by a large separation of anodic and cathodic peak potentials (Figure 1B). The peak potentials for the reductive waves, E_{pc}, range from −892 to −1160 mV (Table 1). In addition, we found that the E_{pc} for the sulfoxide analogue was ca. 500 mV lower than the E_{pc} for the corresponding selenoxide. To the best of our knowledge the data in Table 1 represent the first redox potentials of selenoxides ever reported.

Incubation of selenoxides with reducing agents such as glutathione (GSH) and ascorbate (AscH⁻) results in the formation of corresponding selenides as evidenced by reverse-phase HPLC (RP-HPLC). It is evident from Table 1 that reduction of selenoxides by GSH is a very rapid process, and similar results

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Table 1. Redox Potentials, Peak Potentials, and Second-Order Rate Constants for a Series of Phenylaminoethyl Selenoxides

| E (vs NHE)[a] (mV)	E_{pc} (vs NHE)[b] (mV)	X	R_1	R_2	k_{GSH}[c] (M⁻¹ s⁻¹)	$k_{AscH⁻}$[d] (M⁻¹ s⁻¹)
+480	−892 ± 8	Se	F	H	3450 ± 10	6.6 ± 0.6
+460	ND	Se	Cl	H	4060 ± 20	5.1 ± 0.6
+445	−995 ± 8	Se	H	CH₃	ND	9.6 ± 0.6
+440	−996 ± 7	Se	H	H	1500 ± 20	10 ± 1
+430	ND	Se	CH₃O	H	2200 ± 30	14 ± 1
+410	−1160 ± 10	Se	HO	CH₃	1100 ± 30	15.0 ± 0.4
[e]	−1544 ± 4	S	H	H	NR	NR

[a] Redox potentials, E, were determined by potentiometric titration in 100 mM sodium acetate, pH 5.5, 25 °C using 10 mM sodium dithionite as the reducing titrant as described in the Experimental Section. Values reported are from triplicate experiments and SEM were <2% in all cases. [b] Peak potentials, E_{pc}, were determined by cyclic voltammetry in 100 mM sodium acetate, pH 5.5, at 25 °C as described in the Experimental Section. Second-order rate constants, k, were determined under pseudo-first-order rate conditions by spectrophotometric detection at [c] 25 °C in 100 mM potassium phosphate, pH 7.0, or at [d] 37 °C in 100 mM MES, pH 5.5. [e] A well-defined titration curve could not be obtained using sodium dithionite. NR = no reaction, ND = not determined.

have been reported with other selenoxides and thiols.[22,23] In contrast, the reaction with AscH⁻ was much slower. In both cases the reactions were first order with respect to selenoxide and with respect to reductant. As expected, reductant:selenoxide stoichiometries of 1:1 were obtained for AscH⁻, and 2:1 for GSH. There was no observable redox reaction between the sulfoxide and the reducing agents examined. Chen et al.[22] have proposed a thioselenurane intermediate for the reaction of methylphenyl-selenoxide with GSH, and our kinetic data for GSH oxidation is consistent with such an intermediate (Scheme 1). In addition, our ρ value (+0.9 ± 0.2) differs markedly from the large negative ρ values reported for nucleophilic attack of

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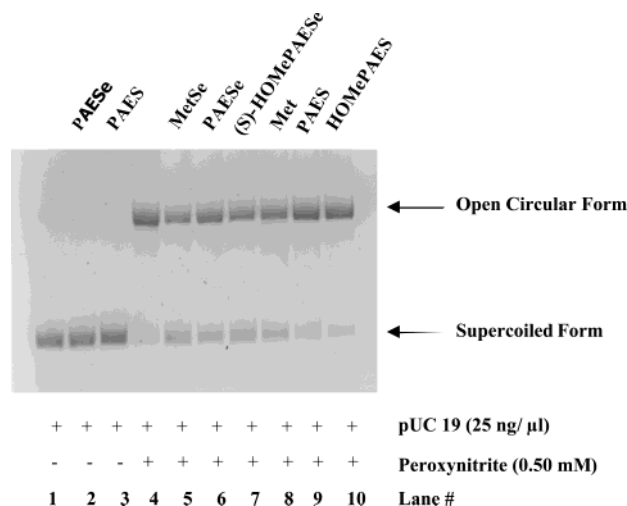
Figure 2. Agarose gel electrophoretic profile of peroxynitrite-mediated damage to pUC19 plasmid DNA in the presence of various selenium- and sulfur-containing compounds. pUC19 plasmid (25 ng/μL) was incubated with peroxynitrite (500 μM) in the presence of various selenium- and sulfur-containing compounds at 500 μM concentration as described in the Experimental Section. The DNA bands were resolved by agarose gel electrophoresis and then scanned with a Molecular Dynamics laser densitometer. In the control reaction peroxynitrite lane 4 causes 88 ± 4% of supercoiled DNA to become nicked; this level of damage was set to 100%. On the figure shown, PAESe, HOMePAESe, and selenomethionine decreased the amount of damaged DNA by 31, 38, and 46%, respectively. By comparison, the sulfur analogues PAES, HOMePAES, and methionine were much less protective against DNA damage (15, 11, and 27%, respectively).

GSH and other thiols on various electrophiles.[24] This is consistent with rate-limiting breakdown of the intermediate, where attack of GSH is not occurring directly on the benzylic "selen-oxy" moiety.

Peroxynitrite has been previously shown to cause single- and double-strand breakage of supercoiled plasmid pUC19 DNA.[12] Since phenylaminoethyl selenides are known to be readily oxidized by peroxynitrite to yield the corresponding selenoxides,[18] we investigated the ability of these selenides to protect pUC19 plasmid DNA against peroxynitrite-induced damage. In a typical experiment, 500 μM peroxynitrite was incubated with pUC19 DNA (25 ng/mL, pH 7.4) for 3 min at 25 °C, and the plasmid forms were then separated using gel electrophoresis and were quantified by scanning (Figure 2). As shown in the figure, the selenium compounds HOMePAESe, PAESe, and selenomethionine decreased peroxynitrite-induced pUC19 DNA damage by as much as 46% under the experimental conditions, and the selenides were much more effective in this regard than the corresponding sulfur analogues. Quantitative RP-HPLC analysis of the reaction mixtures confirmed stoichiometric formation of corresponding phenylaminoethyl selenoxides and sulfoxides as the sole products of the peroxynitrite-mediated oxidation reactions.

MatLab simulations were then employed to model the feasibility of selenide regeneration from the product selenoxide by GSH in the presence of peroxynitrite at physiological pH. Modeling was carried out for the most effective phenylaminoethylselenide, HOMePAESe, using the second-order rate constants for the reactions of HOMePAESe and peroxynitrite (3010 M^{-1} s^{-1}),[18] HOMePAESeO and GSH (1100 M^{-1} s^{-1}; Table

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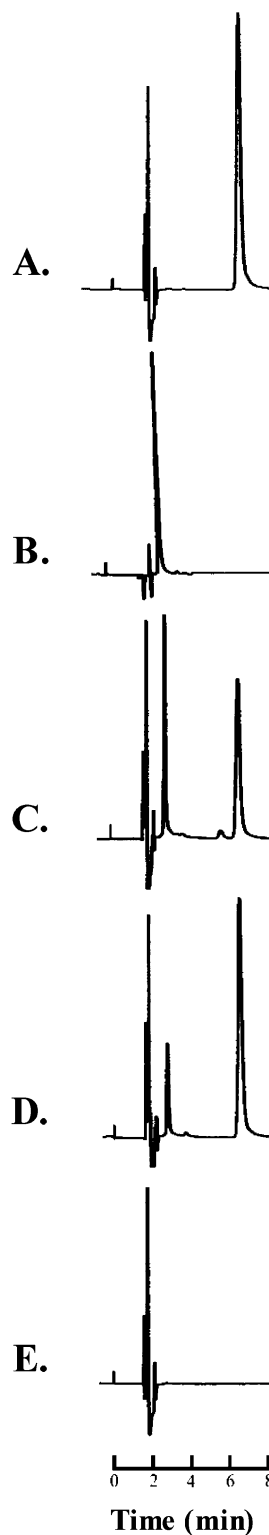
Figure 3. HPLC profiles of pUC19 plasmid assays. Peroxynitrite-mediated reactions with pUC19 plasmid were carried out as described in the Experimental Section and analyzed by HPLC with UV detection at 236 nm. Separation of the selenium-containing products and reactants was accomplished on a C-8 reverse phase column with a mobile phase of 15% acetonitrile, 85% water, and 0.1% TFA at a flow rate of 1.5 mL/min. (A) Chromatogram of authentic HOMePAESe, retention time of approximately 7 min. (B) Chromatogram of authentic HOMePAESeO, retention time of approximately 3 min. (C) Reaction mixture containing 25 ng/μL pUC19 plasmid, 250 μM peroxynitrite, and 250 μM HOMePAESe. (D) Reaction mixture containing 25 ng/μL pUC19 plasmid, 250 μM peroxynitrite, 250 μM HOMePAESe, and 250 μM GSH. (E) Reaction mixture containing 25 ng/μL pUC19 plasmid, 250 μM peroxynitrite, and 250 μM GSH.

Scheme 1. Selenium Recycling in the Presence of Cellular Oxidants[a]
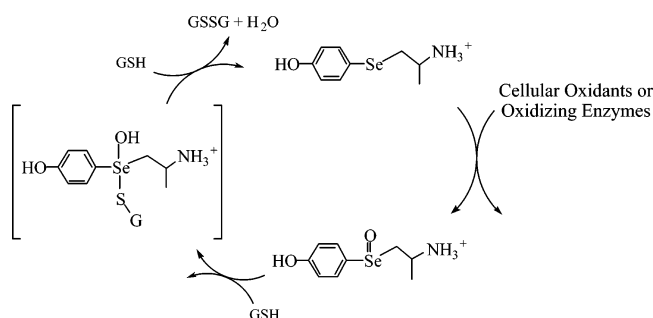
[a] Recycling is initiated by oxygenation of the selenide, followed by reduction of the selenoxide back to the selenide, with stoichiometric oxidation of reduced glutathione. This may occur with the intermediacy of a species such as a thioselenurane.

1), and GSH and peroxynitrite (580 M^{-1} s^{-1})[25] at 25 °C. The apparent first-order rate constant for the decomposition of peroxynitrite under these conditions was also taken into account (0.5 s^{-1}).[18] The MatLab simulations showed that the extent of recycling (i.e., the fraction of selenoxide converted back to selenide before all the peroxynitrite is consumed) was approximately 25% when equivalent concentrations (250 μM) of GSH, selenide, and peroxynitrite were used. Simulations were carried out using the physiological concentration range of GSH (0.1−10 mM)[26] and 250 μM concentrations of selenide and peroxynitrite, and the extent of recycling ranged from 5 to 100%.

With these feasibility modeling results in hand, experiments were then carried out to demonstrate directly that selenium redox cycling by GSH enhances protection of DNA against peroxynitrite-induced damage. In the presence of 250 μM HOMe-PAESe, the amount of DNA damage caused by 250 μM peroxynitrite was reduced by 31% ($n = 6$), as compared to the level of damage in the absence of any protecting agents. Addition of 250 μM GSH to the HOMePAESe reaction caused a statistically significant enhancement of protection of up to 14.5%. An ANOVA/Dunnet test ($n = 6$) was used to confirm that the results of the protection studies were statistically significant. Neither HOMePAESeO nor GSH alone at this concentration provided protective effects against DNA damage. The occurrence of recycling was confirmed by the observed reduction in the selenoxide concentration and the stoichiometric increase in the selenide concentration using RP-HPLC (Figure 3). Others have investigated inhibition of the peroxynitrite-mediated oxidation of dihydrorhodamine by selenomethionine (SeMet) and have reported additive effects of up to 16% upon addition of GSH.[27]

Recently, evidence has emerged that the catalytic redox cycle of mammalian TR involves formation of a Cys-SeCys moiety near the C-terminus of this enzyme, and that this Cys-SeCys receives electrons from a pair of Cys residues located near the N-terminus of mammalian TR.[28−31] This implies that the two-electron reduction potential of the Cys-Cys disulfide is more negative than that of Cys-SeCys. Besse et al.[32] have reported that the redox potential of a SeCys-Cys-peptide (−326 mV at pH 7 and 20 °C) is much lower than that of the Cys-Cys-peptide (−180 mV). From data in Table 1, we calculate the redox potentials of selenoxides at pH 7.0 to be ca. +350 mV; this is much higher than that of the selenenylsulfide-bridged peptide. Therefore, on thermodynamic grounds, a species more closely resembling a selenoxide (or a thioseleninate) would be an attractive candidate for an oxidized selenium moiety generated during selenoenzyme catalysis.[33−35] However, at least in the case of mammalian TR, the evidence in favor of the selenenyl-sulfide[30,31] indicates that its redox potential, and/or that of the Cys-Cys disulfide moiety, is markedly altered in the active-site environment of this enzyme.

There is much interest in the protective roles of antioxidants against oxidant-induced tissue damage.[36−39] Our results indicate that, in addition to the beneficial antioxidant activity of selenoenzymes, exogenously supplied or metabolically generated organoselenium compounds, functioning via a redox cycle such as that illustrated in Scheme 1, may be capable of supplementing natural cellular defenses against oxidative DNA damage.

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