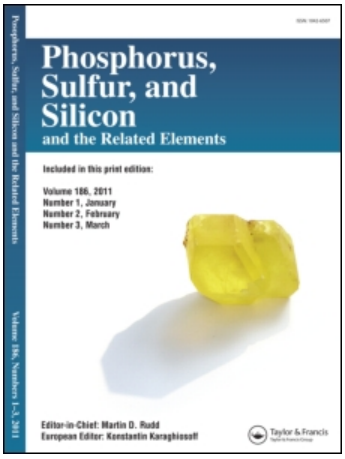

Phosphorus, Sulfur, and Silicon and the Related Elements

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

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To cite this Article Mishra, Beena , Barik, Atanu , Kunwar, Amit , Kumbhare, Liladhar B. , Priyadarsini, K. Indira and Jain, Vimal K.(2008) 'Correlating the GPx Activity of Selenocystine Derivatives with One-Electron Redox Reactions', Phosphorus, Sulfur, and Silicon and the Related Elements, 183: 4, 1018 — 1025

To link to this Article: DOI: 10.1080/10426500801901046

URL: http://dx.doi.org/10.1080/10426500801901046

Phosphorus, Sulfur, and Silicon, 183:1018–1025, 2008

ISSN: 1042-6507 print / 1563-5325 online
DOI: 10.1080/10426500801901046

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Correlating the GPx Activity of Selenocystine Derivatives with One-Electron Redox Reactions

Beena Mishra,[1] Atanu Barik,[1] Amit Kunwar,[1] Liladhar B. Kumbhare,[1] K. Indira Priyadarsini,[1] and Vimal K. Jain[2]

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With an aim to develop water soluble, less toxic glutathione peroxidase (GPx) mimics, three selenocystine (SeCys) derivatives, viz., selenocystamine (SeA), diselenodipropionic acid (SeP), and methyl ester of diselenodipropionic acid (MeSeP) have been synthesized and examined for GPx activity along with SeCys. The GPx activity of the compounds was found to be in the order SeCys ≅ SeA > MeSeP > SeP. The relative affinity of these GPx mimics towards the substrates thiol and hydroperoxide were determined by Lineweaver-Burk (L-B) plots. Since the enzyme activity involves several steps of reduction and oxidation reactions, attempts have been made to understand the role of such processes in deciding the efficacy of diselenides as GPx mimics. For this, one-electron redox chemistry of these compounds was studied in aqueous solutions at pH 7 using nanosecond pulse radiolysis technique. From these studies, it was concluded that SeCys and SeA, which can undergo easy one-electron reduction, exhibit high GPx activity.

Keywords Selenocystine; diselenides; GPx activity; pulse radiolysis; redox reactions

INTRODUCTION

Selenium is an essential micronutrient playing a crucial role in the biochemistry of redox active proteins that are involved in many physiological functions. At least more than thirty proteins have been identified in mammals which involve selenium as active center. One of the most studied selenoproteins is glutathione peroxidase (GPx), which is an antioxidant enzyme that neutralizes reactive oxygen species like hydroperoxides and peroxynitrite at the expense of thiol.[1] Of late, there is

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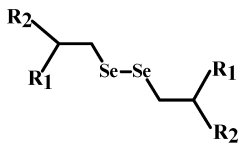
a growing interest in developing new organoselenium compounds having GPx-like activity. Recently several low molecular weight organoselenium compounds have been tested for the GPx enzyme mimicking activity. One such promising compound was ebselen, an aromatic selenide, showing very efficient GPx activity which was even tested in the clinic.[1] Even among the most effective GPx mimics, the therapeutic utility remained limited due to their low water solubility and high cellular toxicity.[1]

Recently, Back et al. showed that certain aliphatic seleno-ethers and diselenides show higher GPx activity than ebselen depending on the substitution on the aliphatic chain.[2] Nauser et al. have showed that the GPx activity of selenocystine depends both on one- and two-electron reduction potential.[3] With this background, and with an aim to develop water-soluble, non-toxic GPx mimics, we have synthesized and tested the GPx activity of three selenocystine derivatives and compared with that of selenocystine. Attempts have also been made to correlate their redox properties with their GPx activity. Here in this paper, all these compounds are collectively termed as diselenides (RSeSeR) and their structures are given in Scheme 1.



	R_1	R_2
Selenocystine (SeCys)	$-COOH$	$-NH_3^+Cl^-$
Selenocystamine (SeA)	$-NH_3^+Cl^-$	H
Diselenodipropionic acid (SeP)	$-COOH$	H
Methyl ester of Diselenodipropionic acid (MeSeP)	$-COOCH_3$	H

SCHEME 1 Chemical Structures of the diselenides (RSeSeR).

MATERIALS AND METHODS

Selenocystine, selenocystamine dihydrochloride, NADPH, glutathione reductase, glutathione, cumene hydroperoxide, methyl viologen and $ABTS^{2-}$ (2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate) ion) were obtained from Sigma/Aldrich. Solutions were prepared using nanopure water from Millipore Elix 3/A-10 system. Diselenodipropionic acid and its methyl ester derivative were synthesized by according to literature method.[4] All the compounds were characterized by ^1H, ^{13}C, ^{77}Se NMR, IR, and elemental analysis. For pulse radiolysis studies, 7 MeV electrons of 500 ns pulse width from a linear accelerator were used.[5]

The transients were detected by absorption spectrometry and the absorbed dose was determined by thiocyanate dosimetry.[5] The reducing formate ($CO_2^{\bullet -}$) radicals and oxidizing trichloromethyl peroxyl ($CCl_3O_2^{\bullet}$) radicals were generated as describe elsewhere.[6]

RESULTS AND DISCUSSION

Enzyme Kinetic Studies

To compare the GPx activity of diselenides, NADPH coupled assay[7] was employed using cumene hydroperoxide (CuOOH) and glutathione (GSH) as the oxidant and reductant respectively by following the decay of NADPH at 340 nm ($\varepsilon_{340\ nm} = 6220\ M^{-1}cm^{-1}$). The reaction mixture contained 300 μM NADPH, 0.4 U/ml glutathione reductase and 50 μM selenium catalyst (except 100 μM SeP) along with varying amounts of GSH and CuOOH. From the initial linear portion of the NADPH decay trace, the initial rate (v) was calculated and this v value is fitted to the Lineweaver-Burk (L-B) equation against varying concentrations of either GSH or CuOOH to estimate the enzyme kinetic parameters, i.e. Michealis-Menten constant (K_m) and V_{max} according to Equation (1).

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

Here, S is either GSH or CuOOH. L-B plot for thiol was obtained by keeping the concentration of CuOOH (180 μM) fixed and varying the concentration of GSH (0.5 mM–17 mM). The linear fit of the data in accordance with equation (1) gave K_m and V_{max} values for thiol which are given in Table I. Similarly, L-B plot for CuOOH was obtained at different concentration of CuOOH (0.023–1.44 mM) keeping the concentration of the GSH (4 mM) fixed and, K_m and V_{max} values were calculated (Table 1).

TABLE I Enzyme Kinetic Parameters for the Diselenides Derived from L-B Plots

	Thiol			CuOOH		
RSeSeR	K_m (mM^{-1})	V_{max} (μM/min)	Turn-over number	K_m (μM^{-1})	V_{max} (μM/min)	Turn-over number
SeCys	0.5	56.0	1.12	64	58.0	1.16
SeA	0.4	29.0	0.58	28	50.0	1.00
SeP	10.2	4.8	0.05	140	2.24	2.24×10^{-2}
MeSeP	0.9	5.6	0.11	280	25.60	0.51

Selenium compounds may enter the GPx cycle in two pathways i.e. either by reduction or oxidation mechanism.[2,8] In the former, the diselenide is reduced by thiol to form a selenol which in turn reduces the hydroperoxide with concomitant formation of selenenic acid that is finally regenerated back to selenol by GSH. In the latter pathway, the selenium compound reacts with the hydroperoxide directly to form the selenenic acid. The relative importance of these two pathways depends on the relative affinity of the selenium compounds for either the thiol (GSH) or the hydroperoxide (CuOOH). This can be understood by estimating the values of K_m and V_{max} determined from the L-B plots. K_m is inversely related to the binding of the substrate to the enzyme, i.e., higher the value of K_m, lower is the binding of the substrate to the enzyme. Comparing the K_m values (Table 1) it can be seen that all the compounds have higher affinity for CuOOH. The SeA, SeCys, and MeSeP show affinity towards thiol also whereas SeP shows least affinity towards the thiol.

Since all these diselenides exhibit GPx activity, it is necessary to compare their relative efficiency in terms of the turn-over number, which is defined as the number of moles of substrate consumed or product formed per mole of the catalyst.[9] Here the substrate is CuOOH and the turnover number is expressed as [Equation (2)]:

$$\text{Turn} - \text{over number} = \frac{V_{max}(\mu M/\,\text{min})}{[\text{SeC}(\mu M)]}. \tag{2}$$

As evident from Table 1, SeCys shows maximum turn over number which is very close to that of SeA, while SeP shows the least activity. Comparing the above-determined K_m values with the turnover number, it can be seen that although SeA shows higher affinity for the thiol, the enzyme activity for SeCys is the maximum.

GPx is an oxidoreductase enzyme; therefore, it undergoes both oxidation and reduction during its catalytic cycle. Thus, the redox properties of organoselenium compounds must contribute to their overall GPx activity. The redox processes can be mediated either by one or two-electron transfer. In order to understand the role of one-electron transfer processes in the overall GPx activity of the diselenides, pulse radiolysis studies have been carried out. Pulse radiolysis is an excellent technique to study and follow one-electron induced redox processes directly.

Pulse Radiolysis Studies

Pulse radiolysis induced one-electron reduction and oxidation studies of these compounds were carried out in aqueous solutions at pH 7.

One-electron reduction was studied by using radiolytically produced e^-_{aq} (standard reduction potential −2.9 V vs. NHE) and a secondary one-electron reductant $CO_2^{\bullet -}$ radical anion with a potential of −1.9 V ($CO_2/CO_2^{\bullet -}$) vs. NHE.[10] The reaction of e^-_{aq} with the diselenides was monitored by following the decay of e^-_{aq} at 700 nm in presence of different concentrations of the diselenide. All the diselenides reacted with e^-_{aq} with bimolecular rate constants of 10^9 to 10^{10} $M^{-1}s^{-1}$. Reaction of $CO_2^{\bullet -}$ with SeCys, SeA, and MeSeP produced transients absorbing in the range of 420–460 nm. From another independent studies and in analogy with pulse radiolysis of disulfides[11], the transient formed on one electron reduction of the above diselenides has been assigned to be the selenyl radical (RSe$^\bullet$) produced by the reactions given below [Equations (3, 4)].

$$R-Se-Se-R \xrightarrow[-e^-]{+CCl_3O_2^{\bullet -}} R-Se-Se-R^{\bullet -} + CO_2 \qquad (3)$$

$$R-Se-Se-R^{\bullet -} \rightarrow R-Se^\bullet + R-Se^- \qquad (4)$$

Similarly, reactions of oxidizing radicals $CCl_3O_2^\bullet$ with these diselenides were studied. Reaction of SeP with $CCl_3O_2^\bullet$ radicals produced a transient absorbing at 560 nm characterized as radical cation of SeP formed by one-electron oxidation. Other diselenides did not show any transient formation indicating that the oxidation of these diselenides is more difficult than SeP. The reaction with $CCl_3O_2^\bullet$ radicals is represented by the Equation (5):

$$R-Se-Se-R + \xrightarrow[-e^-]{+CCl_3O_2^{\bullet -}} R-Se-Se-R^{\bullet +}$$

$$\xrightarrow{OH^-} R-Se-OH + R-Se^\bullet. \qquad (5)$$

The above studies with $CO_2^{\bullet -}$ and $CCl_3O_2^\bullet$ radicals indicate that while SeA is easy to reduce, SeP is oxidized easily. The ease of a compound to undergo reduction or oxidation can be predicted from the rate constant for a given reaction. Therefore the bimolecular rate constants for the reaction of $CO_2^{\bullet -}$ and $CCl_3O_2^\bullet$ radicals with the diselenides were estimated by competition kinetics employing methyl viologen (MV^{2+}) as a reference solute for reduction and $ABTS^{2-}$ as the reference for the oxidation. The competing reaction between the diselenide and the standard can be represented by the Equations (6, 7):

$$R^\bullet + RSeSeR \xrightarrow{k_1} \text{Product 1}, \qquad (6)$$

$$R^\bullet + STD(MV^{2+}/ABTS^{2-}) \xrightarrow{k_2} \text{Product 2}(MV^{\bullet +}/ABTS^{\bullet -}), \qquad (7)$$

TABLE II Bimolecular Rate Constants for the Reactions of Reducing and Oxidizing Radicals with Diselenides from Pulse Radiolysis Studies

RSeSeR	k_1 (RSeSeR+$CO_2^{\bullet-}$) $M^{-1}s^{-1}$	k_1 (RSeSeR+$CCl_3O_2^{\bullet}$) $M^{-1}s^{-1}$
SeCys	2.1×10^9	Nd
SeA	3.4×10^9	8.3×10^6
SeP	8.4×10^8	1.7×10^8
MeSeP	9.6×10^8	1.1×10^8

where $R^{\bullet}$ is $CO_2^{\bullet-}$ in case of reduction, $CCl_3O_2^{\bullet}$ in case of oxidation, and STD is the standard reference solute, which is either MV^{2+} or $ABTS^{2-}$ for reduction and oxidation, respectively. The k_1 is the bimolecular rate constant for the reaction between the diselenide and reductant/oxidant while k_2 is the bimolecular rate constant for the reaction between the reference solute and reductant/oxidant. The k_1 value for the reaction can be calculated from the Equation (8):

$$\left(\frac{A_0}{A} - 1\right) = \frac{k_1}{k_2}\frac{[RSeSeR]}{[STD]}, \tag{8}$$

where A_0 and A represent the absorbance (at 605 nm for $MV^{\bullet+}$ and 645 nm for $ABTS^{\bullet-}$radical) in the absence and presence of diselenide at different concentration, respectively. Using this competition kinetics studies, the bimolecular rate constants for the reaction between the diselenide and $CO_2^{\bullet-}$ radicals were determined (Table II). The results indicate that SeA and SeCys show higher reactivity towards the reducing radical $CO_2^{\bullet-}$ while SeP shows the least and therefore is difficult to participate in reduction.

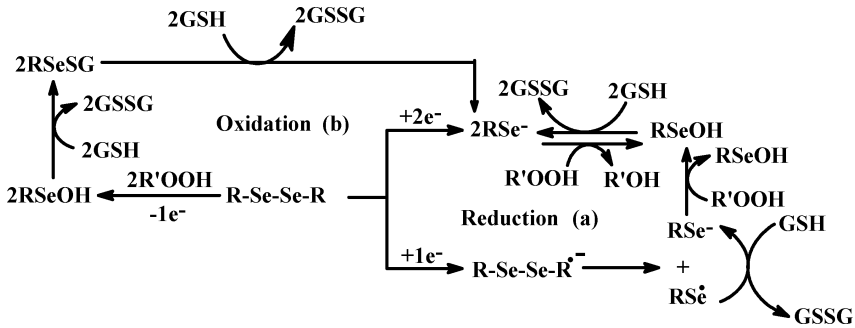
Similarly, the bimolecular rate constant for the reaction between the diselenide and $CCl_3O_2^{\bullet}$ was determined by competition kinetics using $ABTS^{2-}$ as reference solute (Table 2). The results clearly show that SeP can be oxidized easily and shows higher reactivity towards the peroxyl radical.

CONCLUSIONS

Four water-soluble diselenides, derivatives of selenocystine, have been examined for GPx activity. All of them showed GPx activity with differences in their efficiency. SeCys and SeA having amino substitution showed highest GPx activity, while SeP having only carboxylic acid substitution showed the least activity. Using L-B plots, the relative affinity of the diselenides towards thiol and hydroperoxide were estimated. The

results indicated that all the diselenides have higher affinity for the hydroperoxides while SeA and SeCys showed affinity to thiol. These observations were further supported by one-electron redox reactions studied by pulse radiolysis. The studies confirmed that the diselenides having amino substitution are easy to undergo reduction due to the presence electron withdrawing amino groups, which exerts –I effect on the Se-Se bond. On the other hand, SeP, due to electron donating carboxylic group shows +I effect and increases the electron density on the Se-Se bond. SeP is therefore difficult to undergo reduction but is easily oxidized. Thus, the results confirm that the diselenides that can easily undergo reduction show efficient GPx activity.

The different pathways responsible for the GPx activity of diselenides are shown in Scheme 2. The diselenide that are easily reduced react with thiol to form selenol and subsequently by reaction with hydroperoxide form selenenic acid that is recycled back on reaction with thiols. Compound like SeP, which is difficult to undergo reduction but gets oxidized easily may show GPx activity by direct reaction with the hydroperoxide to form the selenenic acid.



SCHEME 2 Possible reaction path followed by the diselenide.

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