

Role of Substrate Reactivity in the Glutathione Peroxidase (GPx) Activity of Selenocystine

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Selenocystine (CysSeSeCys), a diselenide, exhibits glutathione peroxidase (GPx) activity, where it catalyses the reduction of hydroperoxides using a thiol co-factor. To understand the relative reactivity of the two substrates, enzyme kinetic parameters, i.e., the turnover number (k_{cat}) and the relative reactivity parameters toward thiol (ϕ_G) and hydroperoxide (ϕ_H), were determined by applying Dalziel kinetics for a bi-substrate model in the presence of hydrogen peroxide (H_2O_2), *t*-butyl hydroperoxide, or α-cumyl hydroperoxide and glutathione or dithiothreitol (DTT_{red}). The intermediates formed during the reaction of CysSeSeCys with H_2O_2 and DTT_{red} were characterized by ^{77}Se NMR spectroscopy. Ab initio calculation at HF/6-31G(d) indicated that the reactions with H_2O_2 are exothermic, while those with DTT_{red} are endothermic. Based on these studies, the GPx activity of CysSeSeCys is likely to be initiated by the reaction with hydroperoxide and in the catalytic cycle, the reaction with thiol is the rate-determining step.

Selenium, an essential micronutrient is present in many redox active proteins mainly in the form of selenocysteine. To date around 25 selenoproteins are known in humans, out of which glutathione peroxidase (GPx) is one of the most studied selenoenzymes.[1] GPx being an oxidoreductase enzyme reduces various hydroperoxides at the expense of thiol and therefore acts as an endogenous antioxidant enzyme. The enzyme has a tetrameric structure, in which each subunit contains a selenocysteine residue.[2] The mechanism of GPx activity in different protein variants is controlled by the redox property of selenocysteine present in the enzyme.[3]

There is growing interest in developing low molecular weight selenium compounds that act as GPx mimics. Several aliphatic and aromatic organoselenium compounds, e.g., ebselen, benzoselenazolinones, selenenamides, selenoethers, various diselenides, selenocysteine derivatives, and peptides,[4–7] have been synthesized and evaluated for GPx activity. The studies so far have focused on the effects of the structure, substitution, and electrophilicity of the selenium atom. However, most of the experiments using selenium compounds as GPx mimics were performed in organic solvents, which may not act as a good model for the compounds with ionizable functional groups.[4] Yasuda et al. have studied the GPx-like enzyme kinetics of selenocystine (CysSeSeCys) (Chart 1) applying Lineweaver–Burk (L–B) plots.[8] However, there is no comprehensive report quantitatively correlating the enzyme kinetics parameters with the energetics of the intermediate steps.

Recently our group has initiated work on understanding the role of redox processes in the antioxidant GPx activity of diselenides like CysSeSeCys and its derivatives having either an amino or carboxylic group, both in solutions and in vitro models.[9–11] The results indicated that CysSeSeCys containing both amino and carboxylic functional groups could undergo

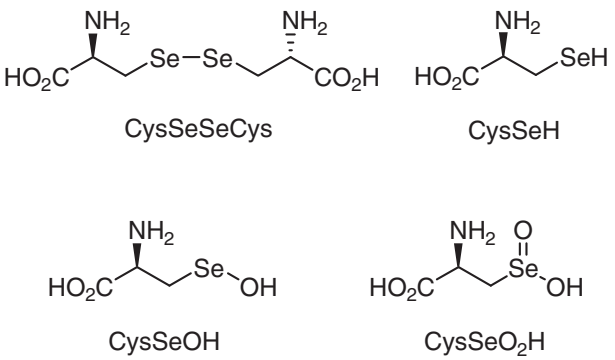

Chart 1. Active intermediates that would be involved in the GPx catalytic cycle of selenocystine (CysSeSeCys). The amino, carboxylic, selenol (–SeH), and seleninic acid ($-SeO_2H$) groups should be ionized in neutral water.

both oxidation and reduction, therefore exhibiting better GPx activity.[9] However, it was not clear which one of the two initial processes is crucial in the catalytic cycle. In order to resolve this, in the present investigation roles of individual substrates involved in the GPx catalytic cycle of CysSeSeCys have been monitored by following enzyme kinetics, ^{77}Se NMR spectroscopy, and quantum chemical calculations.

Results and Discussion

Kinetic Analysis. A diselenide like CysSeSeCys enters the GPx catalytic cycle by two pathways, either by reduction or by oxidation.[4] In the reduction pathway, a diselenide reacts with thiol (RSH) to form selenol (CysSeH), which in turn reduces hydroperoxide through the formation of selenenic acid (CysSeOH). This CysSeOH is converted back to CysSeH, through the intermediacy of selenenyl sulfide (CysSeSR). In the oxidation pathway, CysSeSeCys directly reacts with hydro-

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peroxide to form CysSeOH and seleninic acid ($CysSeO_2H$),[12] and $CysSeO_2H$ should react with three molecules of RSH to produce CysSeSR and RSSR.[13] The preference between the two pathways would depend upon the reactivity of the diselenide with the thiol or the hydroperoxide. These reactions are represented by eqs 1–6.

$$1/2CysSeSeCys + H_2O_2 \xrightarrow{k_1} 1/2CysSeOH + 1/2CysSeO_2H + 1/2H_2O \quad (1)$$

$$1/2CysSeSeCys + RSH \xrightarrow{k_2} CysSeH + 1/2RSSR \quad (2)$$

$$CysSeH + H_2O_2 \xrightarrow{k_3} CysSeOH + H_2O \quad (3)$$

$$CysSeOH + RSH \xrightarrow{k_4} CysSeSR + H_2O \quad (4)$$

$$CysSeSR + RSH \xrightarrow{k_5} CysSeH + RSSR \quad (5)$$

$$CysSeO_2H + 3RSH \xrightarrow{k_6} CysSeSR + RSSR + 2H_2O \quad (6)$$

Enzyme kinetics is a useful tool for understanding the role of these pathways in the actual catalytic cycle of CysSeSeCys. So far researchers have been using L–B plots, which are suitable for single substrate models.[14] However as the GPx catalytic activity involves two substrates, the kinetic data need to be treated with the Dalziel equation, as applied for several multi-substrate models.[15,16] Accordingly, in the present study, the enzyme kinetic parameters were estimated using eq 7.

$$\frac{[E_t]}{\upsilon} = \phi_0 + \frac{\phi_G}{[Thiol]} + \frac{\phi_H}{[H_2O_2]} + \frac{\phi_{GH}}{[Thiol][H_2O_2]} \quad (7)$$

Here $[E_t]$ is the total enzyme concentration. CysSeSeCys being a diselenide produces two reactive species either during oxidation or reduction that can independently participate in the GPx-like cycle (eqs 1 and 2). Therefore, in this case the total enzyme concentration is twice the concentration of CysSeSeCys. In eq 7, ϕ_G is the reciprocal of the total reactivity of the enzyme and its intermediates with thiol [$\phi_G = 1/k_G = 1/(k_2 + k_4 + k_5 + k_6)$], ϕ_H is the reciprocal of the total reactivity of the enzyme and its intermediates with hydroperoxide [$\phi_H = 1/k_H = 1/(k_1 + k_3)$], and ϕ_{GH} is the parameter related to the rate of formation of the ternary complex. The values k_G and k_H are the apparent rate constants for the overall reactions involving thiol and hydroperoxide, respectively. A nonzero value of ϕ_{GH} indicates formation of a ternary complex between CysSeSeCys and both the substrates. ϕ_0 is equal to the reciprocal of the turnover number which corresponds to the maximum catalytic rate at unit enzyme concentration ($\phi_0 = 1/k_{cat}$). To estimate these parameters, a series of experiments were performed and the initial reduction rate of hydrogen peroxide (H_2O_2) (υ) was measured in the presence of CysSeSeCys (0.1 mM) at a fixed initial concentration of thiol [either glutathione (GSH) or dithiothreitol (DTT_{red}) (Chart 2)] and different concentrations of H_2O_2 (45–700 μM). A linear plot was obtained for the variation of $[E_t]/\upsilon$ as a function of the reciprocal concentration of H_2O_2. The slope and intercept of the plot are represented by eqs 8 and 9.

$$\text{Slope} = \phi_H + \frac{\phi_{GH}}{[Thiol]} \quad (8)$$

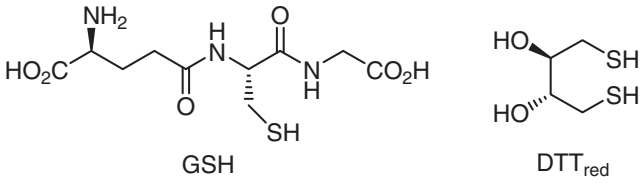


Chart 2. Thiol substrates investigated as a reductant in the GPx catalytic reaction. The amino and carboxylic groups should be ionized in neutral water.

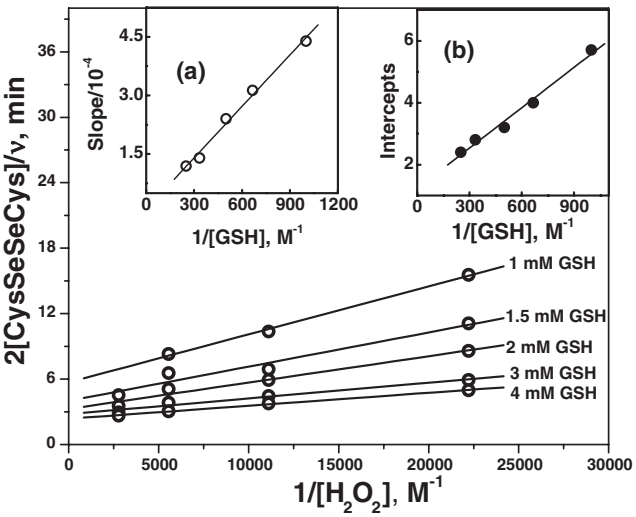


Figure 1. Representative double-reciprocal plots according to Dalziel equation (eq 7) for CysSeSeCys-catalyzed reduction of H_2O_2 in the presence of different concentrations of GSH. Insets (a) and (b) show secondary Dalziel plots in accordance with eqs 8 and 9, respectively.

$$\text{Intercept} = \phi_0 + \frac{\phi_G}{[Thiol]} \quad (9)$$

Similar experiments were repeated with different concentrations of thiol from 1.0 to 4.0 mM, at a variable concentration of H_2O_2 (45–700 μM) and a fixed concentration of CysSeSeCys (0.1 mM), to obtain different slopes and intercept values. The primary plots from all these individual studies did not appear to be parallel (Figure 1 and Figure S1), further suggesting the possibility of the ternary complex formation.[15,16] Therefore, the slope values obtained from these primary plots were plotted as a function of the reciprocal thiol concentration (i.e., [GSH] or $2[DTT_{red}]$) according to eq 8, and from the slope and intercept of this secondary plot, ϕ_{GH} and ϕ_H values, as listed in Table 1, have been derived (Inset (a) of Figure 1). Similarly, the different intercept values obtained from the primary plots at different concentrations of thiol, when plotted against the reciprocal concentration of thiol, gave a secondary plot (Inset (b) of Figure 1). The slope and intercept of this secondary plot, according to eq 9, correspond to ϕ_G and ϕ_0, respectively, as listed in Table 1. The ϕ_G values for the two thiol substrates indicate that the reactivity of CysSeSeCys and its intermediates is higher with DTT_{red} than that with GSH. Comparison of the ϕ_H values with ϕ_G values (Table 1) confirms the higher reactivity of CysSeSeCys and the intermediates with H_2O_2 than that with either of the thiols.

Table 1. Kinetic Coefficients and Apparent Rate Constants of CysSeSeCys for the Reduction of Hydroperoxides by Thiol[a)]

Peroxides	Thiols	ϕ_0 /min	k_{cat} /min^{-1}	$\phi_H \times 10^5$ /M min	$k_H \times 10^{-4}$ /M^{-1} min^{-1}	$\phi_G \times 10^3$ /M min	$k_G \times 10^{-2}$ /M^{-1} min^{-1}	$\phi_{GH} \times 10^7$ /M^2 min
H_2O_2	GSH	1.23	0.81	1.9	5.2	4.3	2.3	4.4
		(±0.12)	(±0.07)	(±0.9)	(±0.4)	(±0.4)	(±0.2)	(±0.5)
	DTT_{red}	0.61	1.63	1.8	5.5	1.9	5.3	1.1
		(±0.05)	(±0.12)	(±0.2)	(±0.5)	(±0.2)	(±0.4)	(±0.1)
CumOOH	GSH	0.88	1.13	9.5	1.0	4.1	2.4	6.1
		(±0.09)	(±0.1)	(±1.2)	(±0.1)	(±0.4)	(±0.1)	(±0.5)
t-BuOOH	GSH	1.64	0.61	20	0.50	7.2	1.3	0.26
		(±0.15)	(±0.05)	(±1.8)	(±0.04)	(±0.7)	(±0.12)	(±0.03)

a) The kinetic parameters listed were obtained by Dalziel analysis using eqs 7–9. See the text for details.

Similarly the reactivity of other hydroperoxides, α-cumyl hydroperoxide (CumOOH) (Figure S2) and *t*-butyl hydroperoxide (*t*-BuOOH) (Figure S3), toward CysSeSeCys was estimated by using GSH as the thiol substrate. The ϕ_0, ϕ_H, ϕ_G, and ϕ_{GH} obtained from these studies are listed in Table 1. As observed above, similar non-parallel primary plots were observed, confirming formation of a ternary complex. Comparison of the ϕ_H values for the three hydroperoxides suggests that the reactivity of CysSeSeCys is the highest with H_2O_2 followed by those with CumOOH and *t*-BuOOH. It can also be confirmed that the reactions of CysSeSeCys and its catalytic intermediates with thiol are slower than those with hydroperoxide.

The reaction of hydroperoxide with CysSeSeCys is initiated by a two-electron transfer process.[17] Therefore, the oxidation of CysSeSeCys would be controlled by redox potentials of the hydroperoxides. Two-electron reduction potential ($E°$) values for H_2O_2, CumOOH, and *t*-BuOOH are 1.76, 1.198, and 1.078 V vs. NHE, respectively.[18] This shows that among the hydroperoxides, H_2O_2 is the strongest oxidizing agent and therefore facilitates the easier electron transfer from CysSeSeCys to H_2O_2 leading to a higher k_H value. Similarly, the reaction with thiols should also be redox controlled as DTT_{ox}/DTT_{red} has a more negative reduction potential (−0.332 V) than GSSG/GSH (−0.240 V).[19,20] Other factors such as structural differences in thiols and hydroperoxides may also significantly control the GPx activities.[21] The large k_{cat} value in the presence of DTT_{red} (1.63 min^{-1}) would be in part due to the dithiol structure which facilitates the conversion from CysSeSR to CysSeH (eq 5) because the reaction becomes a unimolecular process. The small k_G value in the presence of *t*-BuOOH (1.3×10^2 M^{-1} min^{-1}) may be due to indirect effects of the sterically bulky *t*-Bu group that are not known at this moment.

^{77}Se NMR Analysis. After estimation of the enzymatic parameters for CysSeSeCys, characterization of the catalytic intermediates was attempted by ^{77}Se NMR spectroscopy. The study was restricted to DTT_{red} as the thiol substrate and H_2O_2 as the hydroperoxide substrate. The reason for selection of these two specific substrates is that unlike other substrates, DTT_{red} and H_2O_2 are simpler compounds and exhibit minimum interference in the NMR spectra. Since CysSeSeCys has limited solubility at pH 7, the NMR experiments were carried out at pH 10, where the selenol (RSeH), seleninic acid ($RSeO_2H$), and thiol (RSH) functional groups should be ionized.

In the first step, CysSeSeCys was treated with a stoichiometric amount (1:1) of DTT_{red}. The ^{77}Se NMR signal of CysSeSeCys at 272 ppm disappeared completely within twenty minutes and a new peak was observed at −242 ppm, indicating quantitative conversion of CysSeSeCys to selenolate ($CysSe^-$) (Figure S4) as also observed earlier by Tan et al.[22] This complete and quantitative conversion can be rationalized by redox potentials of the individual couples for the equilibrium shown in eq 10.

$$\text{CysSeSeCys} + \text{DTT}_{red} \leftrightarrows 2\text{CysSe}^- + \text{DTT}_{ox} + 2\text{H}^+ \quad (10)$$

The reduction potential values for SeCys ($E_{CysSeSeCys/2CysSe^-,H^+}$) and DTT ($E_{DTTox/DTTred}$) at pH 7 are −0.383 and −0.332 V vs. NHE, respectively,[19] indicating that the reaction should not be spontaneous. However, the reduction potential of DTT would decrease to −0.510 V at pH 10 because the pK_a values for the thiol groups are 9.26 and 10.34. This favorable potential change would be responsible for quantitative conversion of CysSeSeCys to $CysSe^-$ under the NMR experiment conditions. As expected, treating CysSeSeCys with 10 molar equivalents of DTT_{red} at pH 1 did not show any CysSeH formation.

In the second step, an equimolar amount of H_2O_2 was added to the above $CysSe^-$ solution. Two signals were observed at 272 and 1183 ppm in the ^{77}Se NMR spectrum, which could be assigned to CysSeSeCys and $CysSeO_2^-$, respectively: the pK_a values for areneseleninic acids are 4–5.[23] The ratio of the yields was 2:1 according to the integrals of the ^{1}H NMR absorptions. The formation of the seleninate anion ($CysSeO_2^-$) is expected to be through the intermediacy of CysSeOH, which being highly reactive would be trapped either by $CysSe^-$ to yield CysSeSeCys[24] or by excess H_2O_2 to yield $CysSeO_2^-$. The assignment of ^{77}Se NMR peak at 1183 ppm to $CysSeO_2^-$ is based on an earlier observation by House et al. for $BocNHCH_2CH_2SeO_2H$ at 1187 ppm at pH 8.0.[25]

In the third step, CysSeSeCys was treated with a stoichiometric amount (1:1) of H_2O_2 and the reaction was monitored for 24 h. The ^{77}Se NMR signal at 272 ppm decreased and a new peak, corresponding to $CysSeO_2^-$, appeared at 1183 ppm (Figure 2). The reaction was completed in three hours without formation of any other products. Under these conditions, the yield of $CysSeO_2^-$ was estimated to be 33% based on the ^{1}H NMR spectral integration data. The result clearly shows that the oxidation of CysSeOH with H_2O_2 is much faster than that of CysSeSeCys (eq 1). When CysSeSeCys was treated with 3

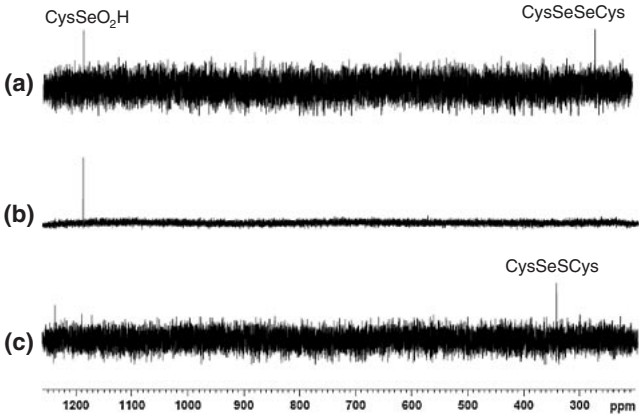


Figure 2. A series of ^{77}Se NMR spectra obtained in D_2O containing NaOH (pH 10) for the reaction mixtures of (a) CysSeSeCys and H_2O_2 (1:1), (b) CysSeSeCys and H_2O_2 (1:3), and (c) CysSeSeCys and H_2O_2 (1:3), then L-CysSH (6 equiv).

equivalents of H_2O_2, the yield of $CysSeO_2^-$ increased up to 88% without formation of any other by-products. $CysSeO_2^-$ was reduced back to CysSeSeCys by the subsequent treatment with DTT_{red} probably through eq 6.[13] However, we could not observe formation of the selenenyl sulfide (CysSeSR) intermediate in the reaction probably because even if it is produced, it may be rapidly converted to cyclic DTT_{ox} and $CysSe^-$, which in turn would react with $CysSeO_2^-$ to produce CysSeSeCys. In another experiment, when a monothiol like L-cysteine (L-CysSH, 3 equivalents), was added to the solution, a signal was however observed at 349 ppm in the ^{77}Se NMR spectrum (Figure 2), indicating formation of a selenenyl sulfide intermediate (CysSeSCys). But no signal corresponding to CysSeOH was observed in any of these NMR experiments.

Ab Initio Calculation. Since CysSeSeCys has ionizable functional groups, the zwitterionic form was employed in the ab initio calculations at HF/6-31G(d). The stable structures for CysSeSeCys, $CysSe^-$, CysSeOH, $CysSeO_2^-$, DTT_{red}, and DTT_{ox} were located in water by exhaustive conformer search, in which all possible dihedral angles were systematically changed and the geometry of each structure was optimized with the PCM model, which is frequently employed for GPx models.[24,26,27] The global energy minimum structures of CysSeSeCys, $CysSe^-$, CysSeOH, and $CysSeO_2^-$ obtained in water are shown in Figure 3. The global energy minimum structure of DTT_{red} was in the extended form, and cyclic DTT_{ox} possessed two hydroxy groups in the equatorial directions in water (Figure S5).

The structures shown in Figure 3 have reasonable bond parameters around the Se atom compared with the structure of GPx enzyme determined by X-ray analysis[28] as well as those calculated for several GPx mimics.[24,26,27,29–32] However, the torsion angle τ(SeC$_\beta$C$_\alpha$C) was in discrepancy with the X-ray structure: Most structures shown in Figure 3 have τ = ca. −60° except for $CysSeO_2^-$ and the right half of the CysSeSeCys model (τ = ca. 180°), while at the active site of GPx the selenocysteine residue has τ = ca. 60°.[28] Nevertheless, the difference in the selenocysteine structure should be due to truncation of the other amino acid residues from the active site

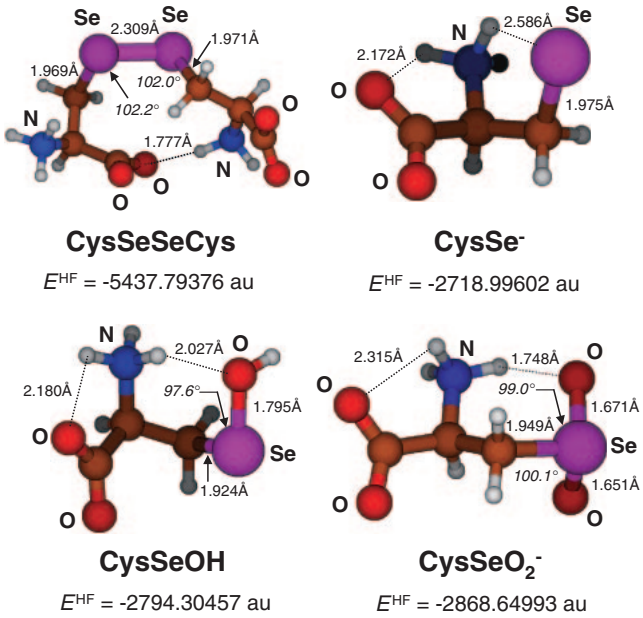


Figure 3. Global energy minimum structures of CysSeSeCys, $CysSe^-$, CysSeOH, and $CysSeO_2^-$ obtained in water at HF/CPCM/6-31G(d). Pertinent atomic distances and bond angles are shown. The torsion angle of CSeSeC in CysSeSeCys was −100.5°.

in our model. The molecular environment around the selenocysteine residue would change the conformation, thereby modifying the antioxidant activity.[27]

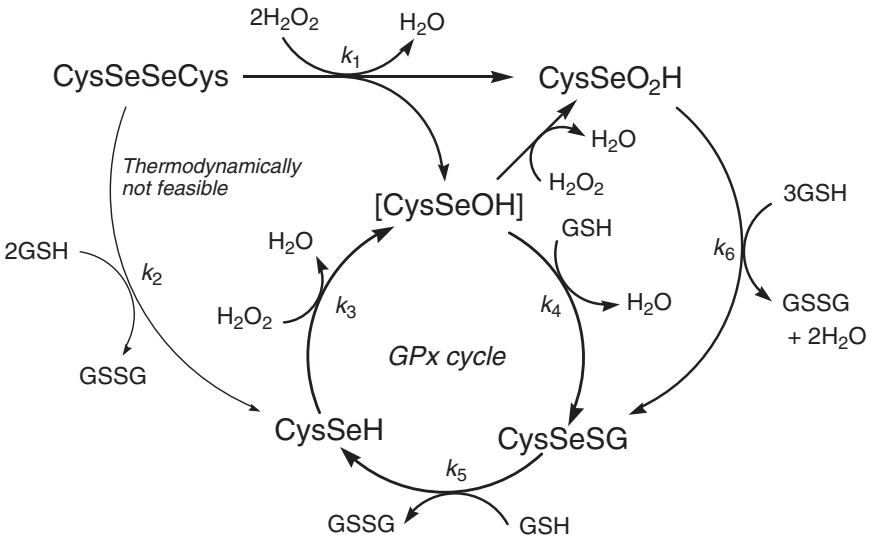
Having obtained the HF energies (E^{HF}) for optimized structures of CysSeSeCys, $CysSe^-$, CysSeOH, $CysSeO_2^-$, DTT_{red}, and DTT_{ox} in water, the reaction energies (ΔE^{HF}) for the most likely steps involved in the GPx cycle have been calculated as shown in Table 2. The results suggest that the reaction of CysSeSeCys with H_2O_2 is exothermic, while that with DTT_{red} is endothermic. Similarly, the reaction of $CysSe^-$ with H_2O_2 is exothermic, while the reaction of CysSeOH with DTT_{red} is endothermic. This indicates that in the catalytic reaction of CysSeSeCys in water, the reaction with hydroperoxide should proceed faster than that with thiol. The results are consistent with the calculation by Bayse,[30] in which three fundamental reactions of the GPx catalytic cycle, i.e., eqs 3–5, were followed by application of an explicit water solvent model for a simple GPx model, benzeneselenol (PhSeH), to reveal that oxidation of the selenol to the selenenic acid with H_2O_2 is exothermic ($\Delta H \approx -60$ kcal mol^{-1}) (1 kcal mol^{-1} = 4.184 kJ mol^{-1}) while reduction of the selenenic acid to the selenol with thiol is much less exothermic ($\Delta H \approx -10$ kcal mol^{-1}).

Further, the global energy minimum structure of CysSeSeCys obtained from quantum chemical calculation does not show any non-bonding interaction between Se and the heteroatoms like O or N. Our observation is contrary to that reported by Yasuda et al. that the GPx-like activity of CysSeSeCys is initiated by reaction with thiol.[8] Support for our observation comes from a recent report by Mugesh et al.,[14,33] where it has been reported that in aromatic diselenides nonbonding interactions assist in the reduction of

Table 2. Energy Changes (ΔE^{HF}) during the GPx-Like Catalytic Cycle of CysSeSeCys in the Reaction of H_2O_2 with DTT_{red}[a)]

Reactions	ΔE^{HF}/kcal mol^{-1}
$CysSeSeCys + DTT_{red} + 2H_2O \rightarrow 2CysSe^- + DTT_{ox} + 2H_3O^+$	94.5
$CysSeSeCys + 2H_2O_2 \rightarrow CysSeOH + CysSeO_2^- + H_3O^+$	−13.8
$CysSe^- + H_2O_2 + H_3O^+ \rightarrow CysSeOH + 2H_2O$	−88.6
$CysSeOH + DTT_{red} \rightarrow CysSe^- + DTT_{ox} + H_3O^+$	25.1

a) Calculated at HF/CPCM/6-31G(d) in water. The global energy minimum structure was employed for each species.



Scheme 1. A plausible catalytic cycle for the GPx-like activity of CysSeSeCys.

diselenides with thiol, while compounds without such interactions react preferably with hydroperoxide rather than thiol.

Conclusion

The GPx-like catalysis of organoselenium compounds utilizes two substrates, hydroperoxide and thiol. The present studies confirm that the GPx-like cycle of CysSeSeCys is initiated preferably by the reaction with hydroperoxide to form CysSeOH and $CysSeO_2^-$, rather than the reaction with thiol to form $CysSe^-$. CysSeOH and $CysSeO_2^-$ subsequently undergo reduction with a thiol substrate to produce CysSeSG, which is converted to the active $CysSe^-$ by the reaction with another thiol molecule. In the overall GPx cycle, the reduction with thiol being the slowest becomes the rate-determining process. All these possible reactions are summarized in Scheme 1. Based on these studies it is suggested that while developing water-soluble GPx mimics, molecular design of a diselenide should be made in such a way that it can be easily oxidized and the resulting intermediates can undergo easier reduction.

Experimental

General. Selenocystine, reduced nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH), glutathione reductase, glutathione (GSH), reduced dithiothreitol (DTT_{red}), hydrogen peroxide (H_2O_2), *t*-butyl hydroperoxide (*t*-BuOOH), and α-cumyl hydroperoxide (CumOOH) from Sigma/Aldrich have been procured from local agents. The concentrations of aqueous H_2O_2 (30% H_2O_2), CumOOH, and *t*-butyl hydroperoxide were estimated by iodometric titration.[34] Solutions were prepared using water from a nanopure system. The purity of CysSeSeCys was verified by ^1H and $^{77}Se\{^1H\}$ NMR spectra recorded on a Bruker AV500 NMR spectrometer operating at 500 and 95.43 MHz, respectively. D_2O (99.8 atom %D) containing a small portion of NaOH (pH 10) was employed as the solvent. Chemical shifts δ are given in ppm as the shifts from $CHCl_3$ (for ^1H) and diphenyl diselenide (for ^{77}Se) as external standards. For the NMR experiments the concentration of CysSeSeCys was 15 mM at pH 10 and all experiments were carried out under inert conditions (argon atmosphere).

GPx Activity. GPx activities of CysSeSeCys were monitored spectrophotometrically by using either NADPH–GSSG reductase coupled assay[9,10] or by the direct oxidation of DTT_{red}.[35] In the former assay, the test mixture contained NADPH, GSH, and glutathione reductase in 0.1 M potassium salts of phosphate buffer (pH 7.4). CysSeSeCys was added to the mixture, and the reaction was initiated by addition of hydroperoxide. The initial concentrations of NADPH, GSH, glutathione reductase, CysSeSeCys, and the hydroperoxide were 0.34 mM, 1.0–4.0 mM, 5.0 mU mL^{-1}, 0.1 mM, and 45–700 μM, respectively. The initial reduction rate of the hydroperoxide (υ) was calculated from the rate of NADPH oxidation by following the decay of absorbance due to NADPH at 340 nm. In the absence of CysSeSeCys, the decay was quite slow. In the second assay, the peroxidase-like activity of CysSeSeCys using DTT_{red} as a substrate was monitored by following the formation of DTT_{ox} at 310 nm both in the

absence and presence of CysSeSeCys. The initial concentrations of DTT_{red}, H_2O_2, and CysSeSeCys were 0.5–4.0 mM, 45–360 μM, and 0.1 mM, respectively. Using υ, the kinetic parameters were estimated by employing Dalziel plots for a bi-substrate model, according to a procedure given in references.[15,16]

Ab Initio Calculation. Quantum chemical calculations were performed by using a Gaussian 03 software package (revision B.04).[36] Stable conformers were systematically searched by changing all the possible dihedral angles in a step size of 120°. Geometry of each built structure was optimized at the HF/6-31G(d) level in water by using a conductor-like solvation model (CPCM), which is a modified form of a polarizable continuum model (PCM).[37,38] In PCM, the solvent is modeled as a continuous static medium characterized by a dielectric constant (ε), which is modified as a scaled conductor boundary in CPCM. The energies were not corrected to the zero-point energies.

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Supporting Information

Additional experimental and calculation results. This material is available free of charge on the Web at http://www.csj.jp/journals/bcsj/.

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