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A Water-Soluble Cyclic Selenide with Enhanced Glutathione Peroxidase-Like Catalytic Activities

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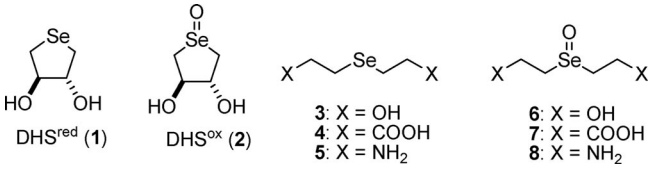
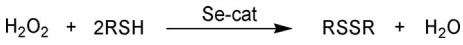
Antioxidative catalytic activities of *trans*-3,4-dihydroxyselenolane (DHS^{red}), a water-soluble cyclic selenide, were investigated in the reaction of hydrogen peroxide with three different thiol substrates, monothiol glutathione (GSH), dithiol dithiothreitol (DTT^{red}), and polythiol reduced ribonuclease A (RNase A) having eight thiol groups along the polypeptide chain. For all the thiol substrates, DHS^{red} exhibited higher glutathione peroxidase (GPx)-like antioxidative catalytic activities than the corresponding linear selenide, that is, $Se(CH_2CH_2OH)_2$. The rate-determining step of the catalytic cycle was unambiguously assigned to the oxidation process of the selenide rather than the reduction process of the selenoxide intermediate. The enhanced catalytic activities of DHS^{red} can be ascribed to the cyclic structure, which elevates the HOMO energy level and makes the selenium atom more exposed to the surroundings.

Introduction

Organic selenides have frequently been applied as synthetic mimics of glutathione peroxidase (GPx), a selenium-containing antioxidant enzyme catalyzing the reduction of hydroperoxides at the expense of two molecules of glutathione (GSH).[1] For example, selenomethionine, a selenium analog of amino acid methionine, was demonstrated to have an antioxidative catalytic activity[2] and has been widely applied as an anticancer reagent or an antioxidant.[3] Bis(3-hydroxypropyl) selenide and the related selenide compounds were recently found to exhibit strong antioxidative catalytic activities as GPx mimics.[4] Redox reactions of many other selenides have also been investigated.[5] We recently synthesized *trans*-3,4-dihydroxyselenolane (DHS^{red}, **1**), a water-soluble cyclic selenide,[6] applied the corresponding selenoxide (DHS^{ox}, **2**) to redox-coupled folding experiments of ribonuclease A (RNase A)[7] and demonstrated that **2** is a powerful oxidant for the rapid and quantitative transformation of the cysteinyl thiol groups into the disulfide (SS) bonds over a wide pH range. Thus, redox chemistry of selenides and selenoxides has attracted increasing interest in relation to their biochemical applications.



In the present study, the GPx-like antioxidative catalytic activities of **1** were investigated in the reactions between hydrogen peroxide (H_2O_2) and various thiol substrates (RSH; Scheme 1). The reaction rates were compared with those obtained by using linear analog **3**[8] as a catalyst to show advantageous features of the cyclic selenide as a redox catalyst. The reactions were also carried out in the presence of selenide catalysts **4**[9] and **5**.[8] The catalytic reaction mechanism as well as the rate-determining step was unambiguously assigned.



Scheme 1. A model reaction to examine GPx-like catalytic activities of selenide catalysts.

Results and Discussion

The GPx-like catalytic activity of **1** was first investigated by using H_2O_2 as an oxidant and glutathione (GSH) as a monothiol substrate in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase (GR). In this assay system, oxidized glutathione (GSSG), which is produced by reaction of GSH with H_2O_2, is reduced by NADPH to GSH with the enzymatic function

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of GR; hence, the reaction progress can be monitored by the decrease in the UV absorbance at 340 nm due to NADPH.[10]

NADPH was completely consumed in ca. 600 s in the absence of a catalyst, as shown in Figure 1. However, it disappeared within 330 s in the presence of **1**. Selenide **3** also showed antioxidative catalytic activity, but the activity was lower than that of **1**, suggesting that a cyclic selenide is more efficient as a GPx mimic than a linear selenide. The catalytic activities of various water-soluble selenides were in the order **4** > **1** > **3** > **5**. The highest activity for **4** would be due to the ionization of the carboxylic groups in the buffer solution to COO^-, the negative charge of which should inductively increase the oxidizability of the selenium atom. Conversely, the lowest activity for **5** would be due to the ionization of the amino groups to NH_3^+, which should decrease the oxidizability of the selenium atom.[11] The order of the catalytic activity strongly suggested that the GPx-like activities of selenides in water can be controlled by the electronic effects from the terminal hydrophilic substituents as well as the steric environments around the selenium atom.

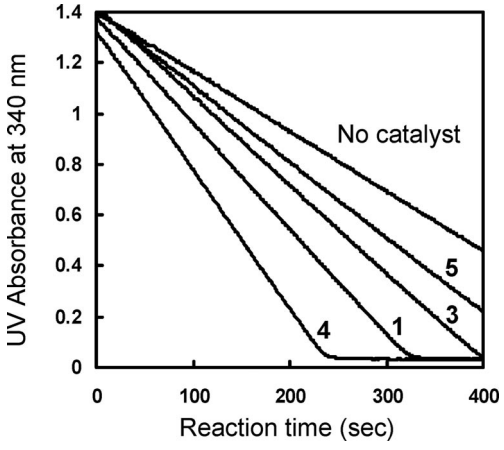

Figure 1. NADPH-coupled GPx assay for water-soluble selenides **1** and **3**–**5**. Reaction conditions: $[GSH]_0$ = 1.0 mM, $[H_2O_2]_0$ = 2.5 mM, $[NADPH]_0$ = 0.3 mM, [GR] = 4 units/mL and [selenide] = 0.2 mM in pH 7.4 phosphate buffer at room temperature.

Second, the catalytic activity of **1** was investigated by using a dithiol, that is, dithiothreitol (DTT^{red}), instead of a monothiol substrate. Methanol (CD_3OD) was selected as a solvent in this assay because the reaction proceeded too fast in water to be monitored by ^1H NMR spectroscopy.[12]

In the series of the ^1H NMR spectra (Figure 2), the absorptions at δ = 2.63, 3.67 ppm due to DTT^{red} decreased with increasing reaction time, whereas those at δ = 2.87, 3.03, 3.49 ppm due to DTT^{ox} increased. In the absence of a catalyst, 86% of DTT^{red} remained unreacted after 300 min (Figure 3). In contrast, the oxidation reaction was complete in 120 min when **1** was added as a catalyst. Again, linear selenide **3** showed a lower catalytic activity than cyclic **1**, suggesting the importance of the cyclic structure. However, the order of the catalytic activity for other selenides, that is, **5** > **1** > **3** ≈ **4**, was very different from that observed in the first assay in water, indicating the presence of strong solvent effects. We found that the high activity for **5** in methanol was due to the free amino groups, which are good base catalysts for the reaction given in Scheme 1.[13] Such a catalytic function of the amino groups should be inhibited in water because of the protonation. On the other hand, the reason for the low catalytic activity for **4** was found to be degradation of the selenoxide intermediate in methanol as discussed later.

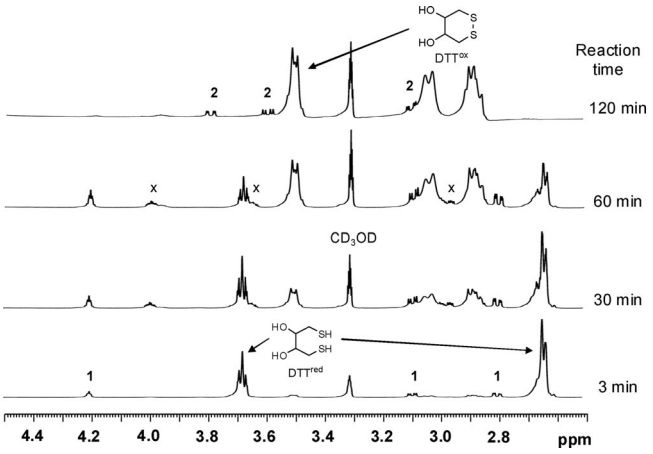

Figure 2. A series of 500 MHz ^1H NMR spectra obtained in the oxidation of DTT^{red} (0.15 mmol) with H_2O_2 (0.15 mmol) in the presence of a catalytic amount of **1** (0.015 mmol) in CD_3OD (1.1 mL) at 25 °C. Unknown byproduct is indicated by ×.

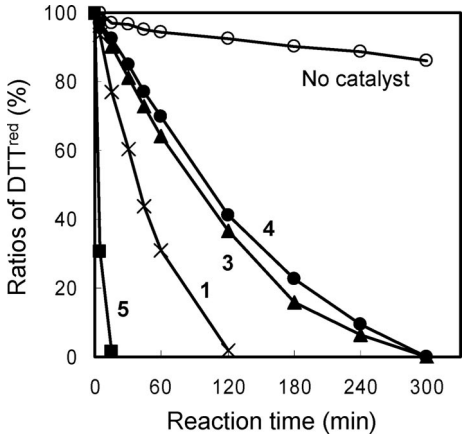

Figure 3. Percentages of residual DTT^{red} as a function of the reaction time in the oxidation of DTT^{red} with H_2O_2 in the presence of selenide catalysts **1** and **3**–**5** in CD_3OD. Reaction conditions: $[DTT^{red}]_0 = [H_2O_2]_0$ = 0.14 mM and [selenide] = 0.014 mM at 25 °C.

Third, reduced RNase A, which has eight thiol groups along the peptide chain, was treated with H_2O_2 in the presence of **1** as a catalyst. RNase A with four native SS linkages is a typical protein that spontaneously folds into the native structure from the reduced unfolded state (**R**) by oxidation and subsequent SS rearrangement.[14] The oxidation reaction was initiated by the addition of H_2O_2 to the acetate buffer solution at pH 4.0 containing **R** and **1** and was quenched by the addition of 2-aminoethyl methanethio-

sulfonate (AEMTS), a thiol blocking reagent.[15] The folding intermediates were then analyzed by HPLC by using a cation exchange column.

The typical HPLC chromatograms are shown in Figure 4. In the presence of catalyst **1** (1 equiv.), SS formation was complete within 180 min, via transient generation of 1S, 2S, and 3S intermediates, corresponding to the intermediate ensembles with one, two, and three SS bonds, respectively, and final generation of a scrambled intermediate ensemble with four SS bonds (4S). On the other hand, in the absence of **1**, formation of SS bonds was very slow with generation of a number of byproducts probably due to side-chain modification.

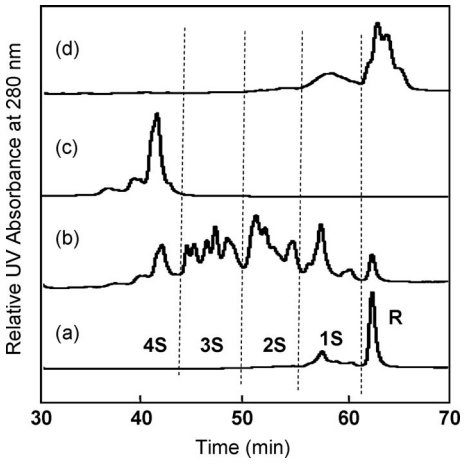

Figure 4. HPLC chromatograms obtained from oxidation of reduced RNase A (**R**) with H_2O_2 catalyzed by **1** at 25 °C and pH 4.0. Reaction conditions: $[R]_0$ = 20 μM, $[H_2O_2]_0$ = 2.0 mM and [**1**] = 20 μM. (a) The reaction time was 10 min, (b) 60 min, and (c) 180 min. (d) The reaction time was 180 min in the absence of the catalyst.

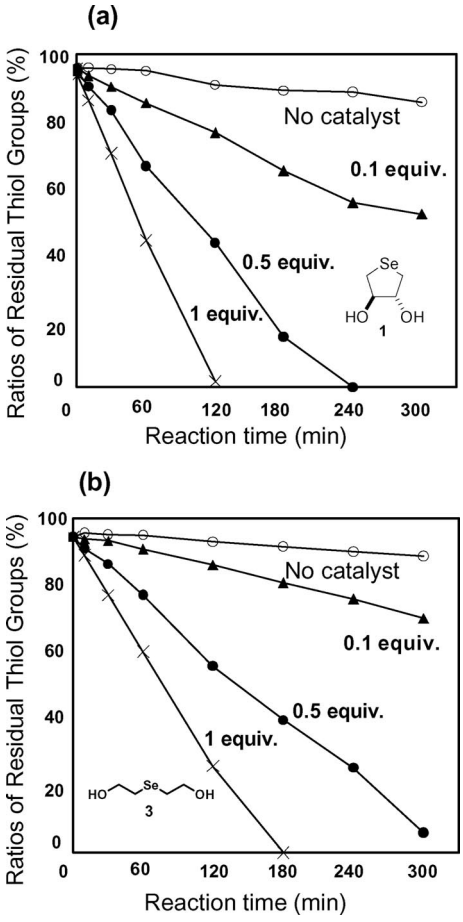

Figure 5. Percentages of the residual cysteinyl thiol groups as a function of the reaction time in the oxidation of reduced RNase A (**R**) with H_2O_2 in the presence of selenide catalysts **1** (top) and **3** (bottom). Reaction conditions: $[R]_0$ = 20 μM and $[H_2O_2]_0$ = 2.0 mM at 25 °C and pH 4.0. Amounts of the catalyst are indicated as the equivalents with respect to **R**.

Figure 5 shows percentages of the remaining cysteinyl thiol groups of **R** as a function of the reaction time in the presence of variable equivalents of catalysts **1** and **3**. When selenide **3** was applied as a catalyst, acceleration of SS formation was also observed, but the catalytic activity was again lower than that of **1**. It should be noted that the activity of the selenide catalyst at neutral or higher pH values was not so obvious as that observed at pH 4.0 because direct oxidation of RSH with H_2O_2 is much faster in basic solutions (see also Figure 1).

The group of Hilvert recently reported that a similar oxidation reaction of **R** by using oxidized selenoglutathione (GSeSeG), a tripeptide dimer with a diselenide bond, as a catalyst finally produced the native folded protein.[16] Our results demonstrated that similar catalytic oxidative protein folding would also be possible by using rather simple water-soluble monoselenides as a catalyst in the presence of peroxides.

The mechanism of the catalytic reaction was delineated by ^{77}Se NMR spectroscopy. When a 1:1 mixture of selenides **1** and **3** was treated with one equivalent of H_2O_2 in D_2O, the absorption due to **1** (δ = 74 ppm) decreased and a new peak, corresponding to selenoxide **2**, appeared at δ = 925 ppm, accompanied with the slight transformation of **3** into **6** (Figure 6). Similar competition experiments revealed the order of reduction abilities for the selenides as **4** > **1** > **3** > **5** in both water and methanol. The order was in complete agreement with their catalytic activities in water. On the other hand, when a 1:1 mixture of isolated selenoxides **2** and **6** was treated with one equivalent of DTT^{red} in D_2O, selenide **1** was quantitatively obtained (Figure 7). The order of oxidation abilities for various selenoxides was revealed to be **8** > **2** > **6** > **7**, which corresponds to **5** > **1** > **3** > **4** for the selenide counterparts. The results clearly demonstrate that only selenide and selenoxide species are involved in the catalytic cycle as the stable intermediates. In methanol, however, selenoxide **7** gradually decomposed into unknown compounds: such degradation products were previously observed in the ^{1}H NMR spectra.[12] The degradation would be responsible for the low catalytic activity of **4** in methanol (see Figure 3) despite the largest reduction ability among the selenides. It is of particular interest that cyclic selenide **1** can be oxidized more easily than linear selenide **3** and resulting cyclic selenoxide **2** can be reduced more easily than linear selenoxide **6**.

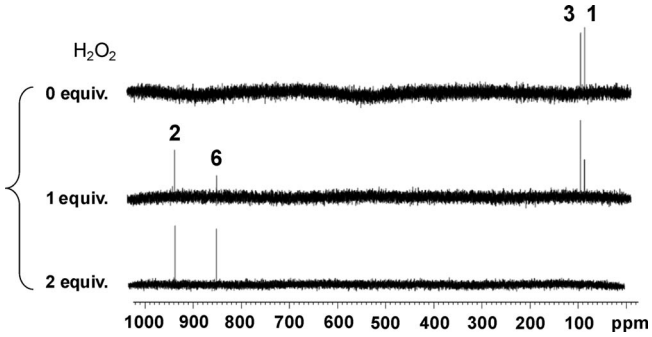

Figure 6. ^{77}Se NMR spectra obtained by addition of H_2O_2 to the solution containing selenides **1** and **3** (0.025 mmol each) in D_2O (1.0 mL) at 25 °C.

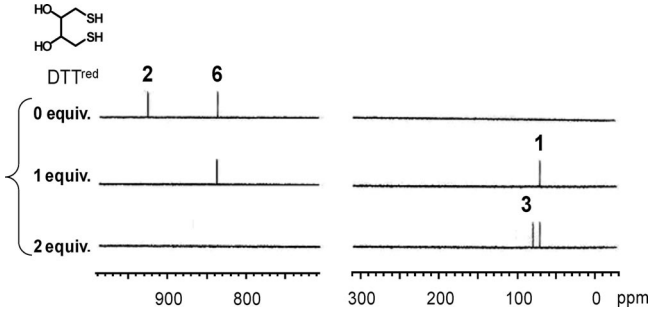

Figure 7. ^{77}Se NMR spectra obtained by addition of DTT^{red} to the solution containing selenoxides **2** and **6** (0.025 mmol each) in D_2O (1.0 mL) at 25 °C.

The above ^{77}Se NMR study strongly suggested that the rate-determining step in the catalytic cycle is the oxidation process from a selenide to the selenoxide. To confirm the assignment, the second-order rate constant (k_{ox}) for the oxidation of **1** with H_2O_2 was determined at 25 °C in water. The obtained value ($k_{ox} = 0.57 \pm 0.03\ M^{-1}s^{-1}$) was significantly smaller than the second-order rate constant for reduction of **2** with a dithiol at pH 8.0 ($k_{red} = 7300\ M^{-1}s^{-1}$), which was estimated from stoichiometric oxidative folding experiments of RNase A by using **2**.[7b]

The enhanced catalytic activity of cyclic **1** can reasonably be explained by properties of the HOMO (Figure 8). Ab initio calculations in water[17,18] revealed that the HOMO energy level of **1** is –8.66 eV, which is 0.27 eV higher than that of **3** in water. Moreover, the HOMO had a dominant contribution from a 4p orbital of the selenium atom: Figure 8 clearly shows that the HOMO of cyclic selenide **1** is completely exposed to its surroundings, permitting easier access of an oxidant, whereas the oxidant access should be slightly hindered for linear selenide **3** due to conformational freedom of the side chains.

Considering all the results, the catalytic mechanism for **1** can be summarized as follows (Figure 9). First, **1** is oxidized with H_2O_2 to the corresponding selenoxide **2**. This step should be the rate-determining step. Second, **2** is rapidly reduced with RSH to produce an intermediate having a Se–S linkage. Because this kind of intermediate could not be observed in any NMR experiments, the intermediate must

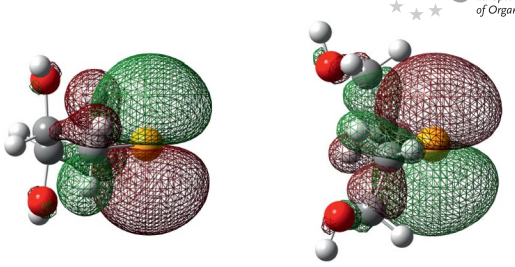

Figure 8. HOMOs of **1** (left) and **3** (right) calculated at HF/6-31G(d). The structures were optimized in water at the same level by using the conductor-like solvation model (CPCM).

be extremely reactive and momentarily capture the second RSH molecule. Similar catalytic cycles would be applicable for **3**–**5** also. In the case of polythiol substrates like DTT^{red} and **R**, the catalytic cycle would proceed more smoothly than the case of a monothiol substrate, because the reaction with a second RSH molecule becomes a unimolecular process.

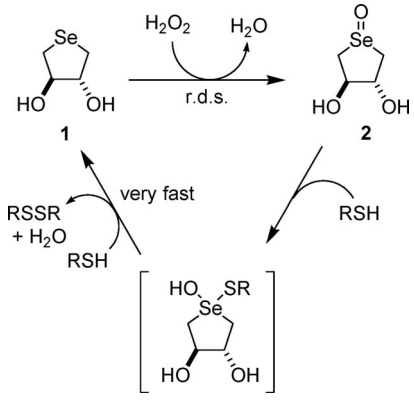

Figure 9. A catalytic cycle of DHS^{red} (**1**) in the reduction of H_2O_2 with RSH.

Conclusions

Water-soluble cyclic selenide **1** exhibited higher GPx-like catalytic activities than linear analog **3** for all mono-, di-, and polythiol substrates. The enhanced catalytic activities can be ascribed to the cyclic structure, which elevates the HOMO energy level and makes the selenium atom more exposed to the surroundings. Although the observed GPx-like catalytic activities may not be so remarkable as those of previous GPx model compounds,[1,4] the features of the water-soluble cyclic selenide will be useful not only for the molecular design of new GPx mimics with enhanced antioxidative catalytic activities but also for biological applications as an efficient catalyst for the formation of SS linkages in proteins.[16]

Experimental Section

General Methods: Selenides **1**,[6] **3**,[8] **4**,[9] and **5**[8] and selenoxide **2**[6] were synthesized according to literature methods. Selenoxides **6**–**8**

were quantitatively obtained in situ within 1 h by the reaction of the corresponding selenides with H_2O_2 in water or synthesized according to the procedure described below for **6** and **8**. Selenoxide **7** was stable only in solution and could not be isolated.[12] Other chemicals and enzymes were purchased from chemical companies and used without purification. ^1H, ^{13}C, and ^{77}Se NMR spectra were recorded at 500, 125.77, and 95.43 MHz, respectively, at 25 °C by using $CHCl_3$, $CDCl_3$, and $(PhSe)_2$ as the external standards for ^1H, ^{13}C, and ^{77}Se, respectively.

Bis(2-Hydroxyethyl) Selenoxide (6): Selenide **3** (30.0 mg, 0.18 mmol) was dissolved in H_2O (1.0 mL). The solution was added with aqueous H_2O_2 (0.18 mmol) at room temperature. After 1 h, the solution was frozen and then lyophilized. The residue was recrystallized from ethanol to give selenoxide **6** as white crystals. ^1H NMR (500 MHz, D_2O, 25 °C): δ = 3.05 (m, 1 H, $SeCH_2$), 3.27 (m, 1 H, $SeCH_2$), 3.94 (m, 2 H, OCH_2) ppm. ^{13}C NMR (125.77 MHz, D_2O, 25 °C): δ = 50.0 ($SeCH_2$), 55.2 (OCH_2) ppm. ^{77}Se NMR (95.43 MHz, D_2O, 25 °C): δ = 838.5 ppm. $C_4H_{10}O_3Se$ (185.08): calcd. C 25.96, H 5.45; found C 26.16, H 5.38.

Bis(2-Aminoethyl) Selenoxide (8): Selenide **5** (140.4 mg, 0.84 mmol) was dissolved in H_2O (1 mL). The solution was added with 1 M HBr (1 mL) and then aqueous H_2O_2 (0.84 mmol) at room temperature. After 1 h, the solution was frozen and then lyophilized. The residue was recrystallized from 2-propanol to give **8**·2HBr·3H_2O as white crystals. ^1H NMR (500 MHz, D_2O, 25 °C): δ = 3.07–3.27 (m, 2 H, $SeCH_2$), 3.37–3.51 (m, 2 H, NCH_2) ppm. ^{13}C NMR (125.77 MHz, D_2O, 25 °C): δ = 34.5 (NCH_2), 41.8 ($SeCH_2$) ppm. ^{77}Se NMR (95.43 MHz, D_2O, 25 °C): δ = 872.2 ppm. $C_4H_{12}N_2OSe$·2HBr·3H_2O (398.98): calcd. C 12.04, H 5.05, N 7.02; found C 11.86, H 4.73, N 7.04.

Activity Measurements: Catalytic activities of the selenides were measured as follows by using GSH, DTT^{red}, and reduced RNase A (**R**) as a thiol substrate according to the literature methods.

In the NADPH and glutathione reductase (GR)-coupled assay,[10] a test solution was prepared by mixing a 100 mM phosphate/6 mM EDTA buffer solution (1941 μL) at pH 7.4 containing NADPH (2.0 μmol) and GSH (6.8 μmol) with a GR solution (453 U/mL, 59 μL). An aliquot (300 μL) of the test solution was added to a 1.0 mM selenide solution (200 μL) in 100 mM phosphate buffer at pH 7.4 and the phosphate buffer solution (430 μL). The reaction was initiated by addition of a 36 mM aqueous H_2O_2 solution (70 μL) to the mixture solution. The reaction progress was monitored by absorption change at 340 nm due to consumption of NADPH.

In the NMR assay,[12] DTT^{red} (0.15 mmol) and selenide (0.015 mmol) were dissolved in CD_3OD (1.1 mL), and the solution was added to 30% H_2O_2 (17 μL, 0.15 mmol) to start the reaction. ^1H NMR spectra were measured at a variable reaction time at 25 °C. The relative populations of DTT^{red} and DTT^{ox} were determined by integration of the ^1H NMR absorptions that were well isolated on the spectra.

In the catalytic oxidation of **R**,[7b] RNase A (5–10 mg) was reduced with DTT^{red} (7–12 mg) in a 100 mM Tris–HCl/1 mM EDTA buffer solution (0.5 mL) at pH 8.0 containing 4 M guanidine thiocyanate as a denaturant. Reduced RNase A (**R**) was desalted to 200 mM acetate buffer at pH 4.0 by using a Sephadex G25 resin column, and the concentration was determined by UV absorbance at 275 nm (ε = 8600 $M^{-1}cm^{-1}$). The **R** solution was immediately diluted with the acetate buffer so that the concentration became 40 μM. An aliquot (200 μL) of the diluted solution was mixed with the acetate buffer solution (100 μL) containing a selenide (8, 40, or 80 μM) and aqueous H_2O_2 solution (100 μL, 8.0 mM). The reaction solution was incubated in a dry thermo bath regulated at 25.0 ± 0.1 °C. After standing for a certain period of time (1 to 300 min), the solution was added to an aqueous AEMTS solution (≈7 mg/mL, 600 μL) to quench the reaction. The collected sample solutions were acidified with acetic acid, desalted into a 0.1 M acetic acid solution and analyzed by HPLC by using a Tosoh TSKgel SP-5PW strong cation exchange column (7.5 × 75 mm). A gradient of sodium sulfate (Na_2SO_4) was applied by changing the ratios of buffer A (25 mM HEPES/1 mM EDTA at pH 7.0) and buffer B (buffer A + 0.5 M Na_2SO_4) from 100:0 to 55:45 over 50 min at a flow rate of 0.5 mL/min. A wavelength of the UV detector was set to 280 nm.

Kinetic Analysis: The velocity of the reaction between H_2O_2 (2.0 mM) and **1** (0.3 mM) was measured at 25 °C in water by following the UV absorption change at 225 nm. The measurement was repeated five times to determine the k_{ox} value.

Ab Initio Calculation: A Gaussian 03 software package (revision B.04)[17] was employed. To obtain the global energy minimum structures for **1** and **3** in water (ε = 78.39), systematic conformer search with geometry optimization was performed in water at HF/6-31G(d) by using the conductor-like solvation model (CPCM).[18] Accuracy of the calculation results was verified by comparing the bond lengths and angles between the calculated global energy minimum structure of **1** and the experimental structure determined by X-ray analysis:[6] the deviations were within ±0.01 Å for the distances and ±0.5° for the angle relevant to the Se atom.

Supporting Information (see footnote on the first page of this article): ^1H, ^{13}C, and ^{77}Se NMR spectra of **6** and **8**; kinetic analysis data of the reaction of DTT^{red} with H_2O_2; Cartesian coordinates of **1** and **3** obtained by ab initio calculations.

Acknowledgments

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