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Modeling the Catalytic Cycle of Glutathione Peroxidase by Nuclear Magnetic Resonance Spectroscopic Analysis of Selenocysteine Selenenic Acids

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ABSTRACT: Although selenocysteine selenenic acids (Sec−SeOHs) have been recognized as key intermediates in the catalytic cycle of glutathione peroxidase (GPx), examples of the direct observation of Sec−SeOH in either protein or small-molecule systems have remained elusive so far, mostly due to their instability. Here, we report the first direct spectroscopic (^{1}H and ^{77}Se NMR) evidence for the formation of Sec−SeOH in small-molecule selenocysteine and selenopeptide model systems with a cradle-type protective group. The catalytic cycle of GPx was investigated using NMR-observable Sec−SeOH models. All the hitherto proposed chemical processes, i.e., not only those of the canonical catalytic cycle but also those involved in the bypass mechanism, including the intramolecular cyclization of Sec−SeOH to the corresponding five-membered ring selenenyl amide, were examined in a stepwise manner.

Glutathione peroxidase (GPx)[1] is one of the most important selenoenzymes[2,3] due to its role in the antioxidant defense in mammalians,[4−6] where it catalyzes the reduction of reactive oxygen species such as H_2O_2.[7] In the widely accepted catalytic cycle of GPx (Process I in Figure 1a), selenocysteine selenenic acids (Sec−SeOHs),[8] generated by the oxidation of selenocysteine selenols (Sec−SeHs), in the catalytic site (Step A in Figure 1a), have been proposed as key intermediates. The Sec−SeH form is assumed to be regenerated by the reaction of the Sec−SeOH intermediate with glutathione (GSH)[9] to produce the selenenyl sulfide (Sec−SeSG) form (Step B),[10] followed by reduction with another molecule of GSH (Step C).

However, examples of the direct observation of Sec−SeOHs in either protein or small-molecule model systems remain elusive. Even the trapping of Sec−SeOHs in a protein by chemical probes has been limited to very few examples.[11−14] One possible reason for this dearth of information is the tendency of Sec−SeOHs to undergo thermal deselenation, which affords the corresponding dehydroalanines (Step D).[15,16] For example, it has been reported that the Sec residues in some selenoproteins (including GPx1) are converted into dehydroalanine in the presence of H_2O_2.[15,17] The mechanism by which GPx maintains its catalytic activity despite the thermal instability of the Sec−SeOH intermediates remains to be determined. Flohé et al. have recently postulated a bypass mechanism to explain the above question (Process II).[18] They proposed that Sec−SeOHs could undergo intramolecular cyclization to the corresponding cyclic selenenyl amides,[19−23] with either a five-membered ring or an eight-membered ring (Step E), to prevent the thermal deselenation (Step D) under GSH-deficient conditions. Their proposal was based on LC-MS/MS analysis of the oxidized forms of bovine GPx1 and rat GPx4, which was found to be consistent with the formula of the cyclic selenenyl amides, and DFT calculations of Process II using a Sec-Gly-Gly tripeptide model. Neither the direct detection nor trapping of the Sec−SeOH forms was achieved in their study. For the chemical elucidation of the mechanistic aspects of the GPx cycle, a model compound for Sec−SeOH with sufficient stability to allow its observation is thus strongly desirable.

In small-molecule model systems, selenenic acids undergo facile dehydrative self-condensation (Figure 1b).[24] Furthermore, selenenic acids generated during the oxidation of selenols readily form diselenides by the reaction with unreacted selenols.[25] Our and other groups have reported on the observation[26−28] and isolation[29−35] of nonselenocysteinyl-derivative selenenic acids by either kinetic or thermodynamic stabilization techniques. However, these nonselenocysteinyl derivatives do not adequately reproduce the characteristic reactivity of Sec−SeOH in proteins.

Recently, we have reported the synthesis of a stable cysteine sulfenic acid that uses a large molecular cavity as a protective cradle.[36] The molecular cradle is also expected to be effective for the protection of reactive selenocysteine derivatives, thus enabling model experiments of reaction processes involving Sec−SeOHs (Figure 1c). Herein, we report the first direct spectroscopic evidence for Sec−SeOHs that are generated via the oxidation of a selenocysteine and selenopeptide bearing the molecular cradle by H_2O_2. The thermal behavior and reactivity of these Sec−SeOHs were investigated using NMR spectros-

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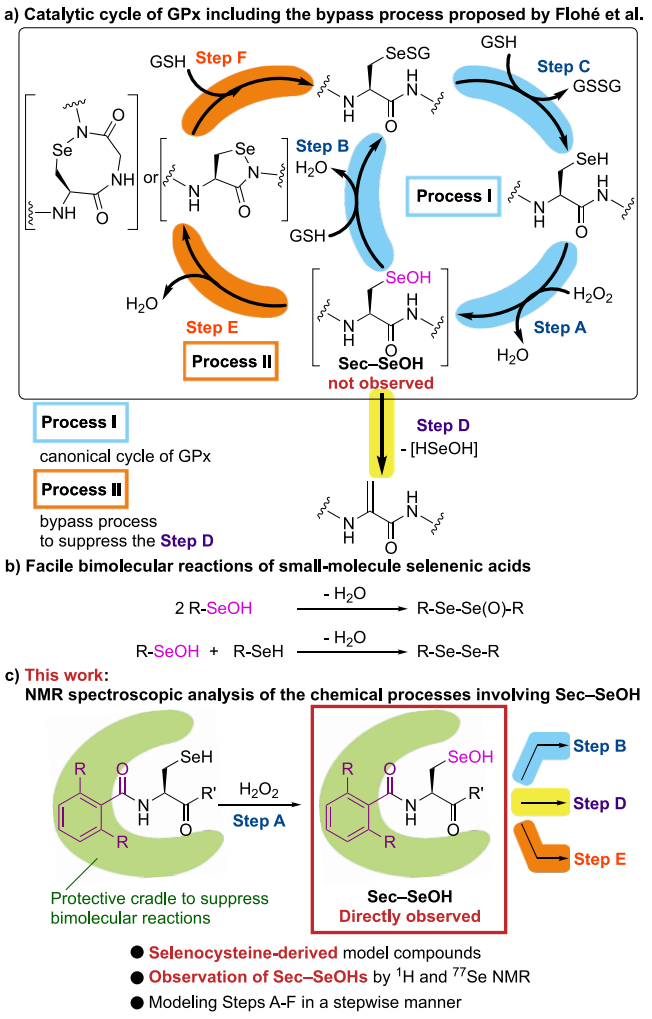


Figure 1. (a) Proposed catalytic cycle of GPx. (b) Bimolecular reactions of selenenic acids. (c) Conceptual illustration of this study.

copy, and all hitherto proposed chemical processes in the catalytic cycle of GPx shown in Figure 1a, including those involved in the bypass mechanism, were documented in a stepwise manner.

We designed two model systems. One is the Sec model (Figure 2A), in which a selenocysteine methyl ester is protected by a cradle-type benzoyl group (henceforth denoted as Bpsc), and the other is the Sec-Gly-Gly model (Figure 2B), which corresponds to the generalized form of the tripeptides in the catalytic site of GPx.[37,38] The Sec–SeOH forms of both

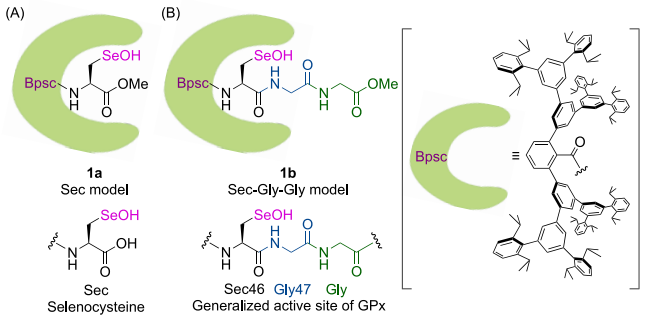
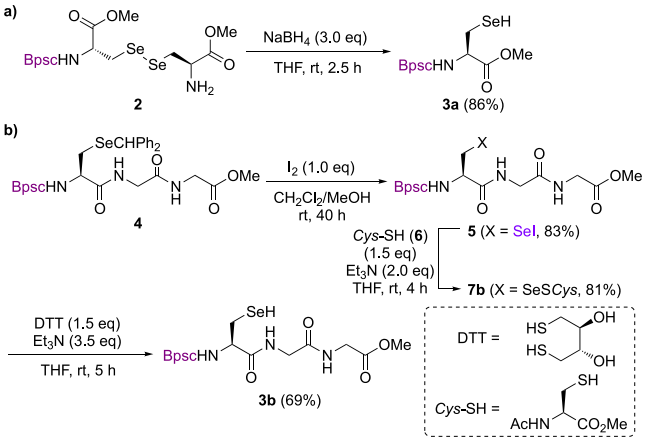


Figure 2. Cradled selenocysteine (A) and selenopeptide (B) model compounds.

models, **1a** and **1b**, are expected to be prevented from bimolecular decomposition (Figure 1b) by the protective cradle.

The Sec–SeH form of the Sec model (**3a**) was synthesized by the reduction of selenocysteine derivative **2** (Scheme 1a).

Scheme 1. Synthesis of the Selenols of the Sec Model (a) and the Sec-Gly-Gly Model (b)



The analogous form of the Sec-Gly-Gly model (**3b**) was synthesized from selenopeptide **4**[39−41] via the route shown in Scheme 1b, which involves selenenyl iodide **5** as a synthetic intermediate.

Selenocysteine-derived selenenyl iodides (Sec–SeIs) have been recognized as important intermediates in the thyroid hormone deiodination by iodothyronine deiodinases,[42,43] albeit that examples of the synthesis of a selenenyl iodide with a selenocysteine backbone remain elusive.[44] Sec–SeI **5** was isolated in the form of reddish-purple crystals, and its structure was determined by a single-crystal X-ray diffraction analysis (Figure 3, Tables S9 and S10).

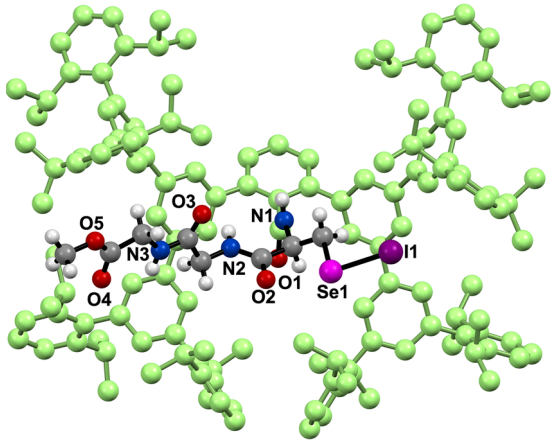


Figure 3. Crystal structure of **5** (one of two independent molecules).

Treatment of Sec–SeH **3a** with H_2O_2 (1 equiv) at room temperature without any additives resulted in the formation of dehydroalanine **8a** in 89% yield together with a small amount of diselenide **9a** (Scheme 2). Although the formation of Sec–SeOH **1a** was not observed directly in this reaction, its intermediacy was supported by trapping experiments using dimedone (**10**) or acetylacetone (**11**)[33] to produce **12a** or **13a**,

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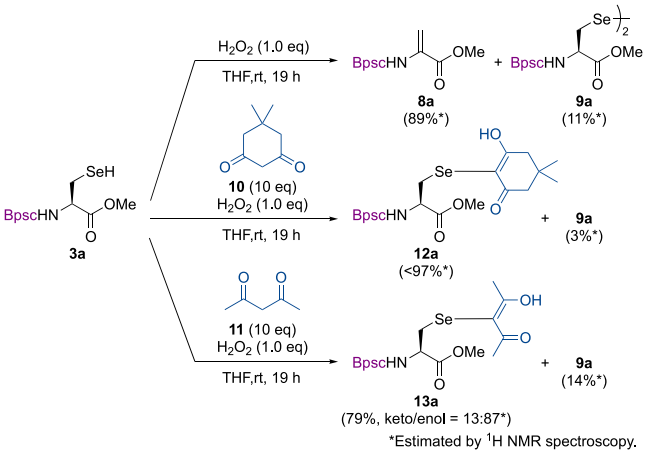
Scheme 2. Oxidation of 3a with H_2O_2 and Trapping Experiments with 1,3-Diketones



respectively. It is likely that, in the absence of trapping agents, the majority of **1a** undergoes thermal deselenation to form **8a**, while a fraction reacted with the substrate **3a** to produce diselenide **9a**. To suppress the formation of **8a** and **9a**, the following protocol was examined (Figure S1). A THF-d_8 solution of **3a** was added dropwise to a THF-d_8/D_2O solution of H_2O_2 (10 equiv) and NaOH[45] at −65 °C. The mixture was transferred to a J-Young NMR tube via a tube cooled to the same temperature, and the ^{1}H and ^{77}Se NMR spectra were recorded at −20 °C (Figure 4a). The formation of Sec−SeOH

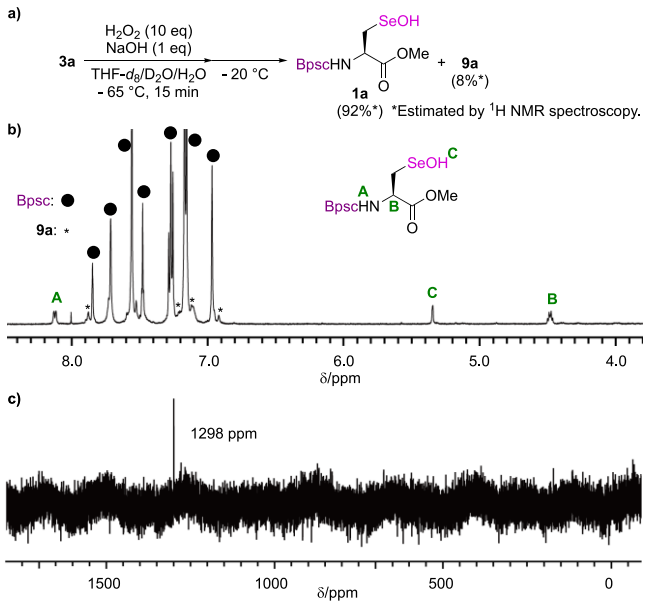
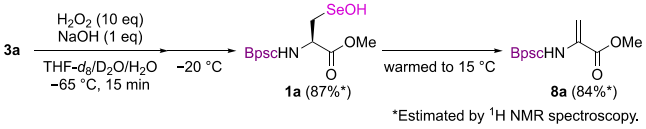


Figure 4. (a) Observation of **1a** via the oxidation of **3a**. (b) ^{1}H NMR spectrum (500 MHz) of **1a** in THF-d_8/D_2O at −20 °C. (c) ^{77}Se NMR spectrum (95 MHz) of **1a** in THF-d_8/D_2O at −20 °C.

1a in 92% NMR yield was observed in the ^{1}H NMR spectrum (Figure 4b).[46] In the ^{77}Se NMR spectrum, **1a** showed a signal at 1298 ppm (Figure 4c), which is in good accordance with those of recently reported arylmethyl-substituted selenenic acids (Table S4).[31,33,47] This is the first experimental evidence for the formation of Sec−SeOHs, as well as the first direct evidence for the oxidation of a Sec−SeH to a Sec−SeOH with H_2O_2 (Step A in Figure 1a). Reactions with 1,3-diketones further confirmed the identification of **1a** as Sec−SeOH. After **1a** was generated by the above protocol, diketone **10** or **11** was added at −20 °C. ^{1}H NMR monitoring indicated that **1a** was gradually converted to **12a** or **13a**, respectively, in good conversion yield (Schemes S18 and S19).

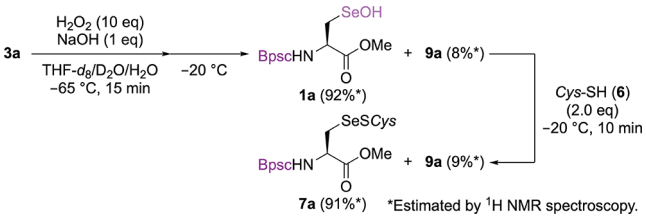
Then, we investigated the thermal deselenation of Sec−SeOH to form a dehydroalanine (Step D). After **1a** was generated by the above protocol, the solution was warmed from −20 to 15 °C. ^{1}H NMR monitoring confirmed that 84% of **1a** was converted into dehydroalanine **8a** (Scheme 3).

Scheme 3. Thermal Deselenation of 1a to 8a



In contrast, when **1a** was generated by the same protocol and subsequently treated with cysteine thiol **6** (2 equiv) at −20 °C, **1a** was rapidly converted into selenenyl sulfide **7a** in 91% conversion yield, and the formation of **8a** was not observed (Scheme 4). These results demonstrate that the reaction of a

Scheme 4. Reaction of 1a with 6



Sec−SeOH with a thiol to produce a selenenyl sulfide (Step B) is rapid, even at low temperature, where the thermal deselenation to a dehydroalanine (Step D) does not proceed.

On the basis of these experiments, the reactivity of a Sec−SeOH is summarized as follows: a Sec−SeOH can be generated as a stable compound by oxidation of a Sec−SeH with H_2O_2 at low temperatures (below −20 °C in the present case). Under these conditions, a Sec−SeOH readily reacts with a thiol to produce the corresponding selenenyl sulfide. However, in the absence of a thiol, it undergoes thermal deselenation to a dehydroalanine upon warming; this reaction can thus be expected to proceed readily at physiological temperature. These properties of a Sec−SeOH strongly suggest that, in the catalytic cycle of GPx, a protective bypass mechanism to prevent its inactivation through deselenation should be operative when the concentration of GSH is insufficient.

Based on the protocol established using the Sec model (Figure 2A), we then conducted model studies of the bypass mechanism (Process II in Figure 1a) using the Sec-Gly-Gly model (Figure 2B). In the oxidation of Sec−SeH **3b** with H_2O_2 using the above protocol, the formation of Sec−SeOH **1b** in 90% NMR yield was observed at −30 °C (Figure 5a and 5b), where **1b** was found to be stable for at least 24 h. In the ^{77}Se NMR spectrum, the signal of **1b** was observed at 1082 ppm (Figure S10). The derivatization of **1b** with acetylacetone (**11**) to produce the corresponding selenide **13b** (Scheme S24) also confirmed the identification of **1b** as a Sec−SeOH. To investigate the intramolecular cyclization of a Sec−SeOH

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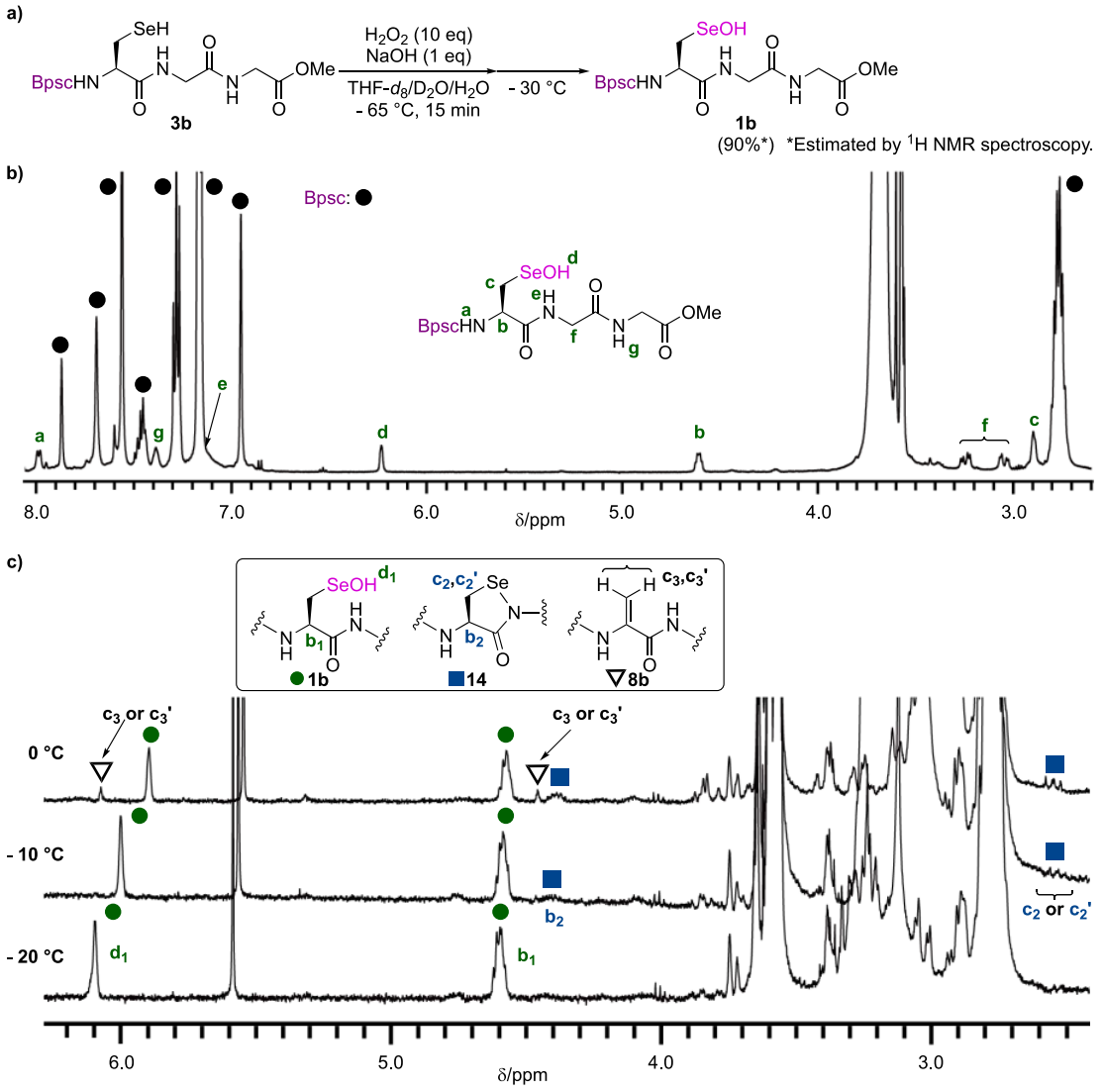


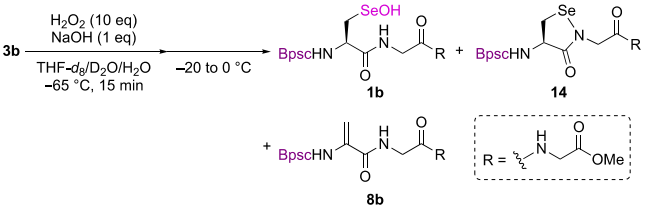
Figure 5. (a) Generation of **1b**. (b) ^{1}H NMR spectrum (500 MHz) of **1b** in THF-d_8/D_2O at −30 °C. (c) VT NMR spectra (400 MHz, − 20 up to 0 °C) for a solution containing **1b** in THF-d_8/D_2O.

to the corresponding cyclic selenenyl amide (Step E), the thermal behavior of **1b** was examined by monitoring the temperature-dependent change in the ratio of **1b** to its products (Figure 5c). When a solution of **1b** generated in 87% NMR yield by the above protocol was warmed from −20 to −10 °C, the formation of cyclic selenenyl amide **14**[48] with a five-membered ring in 6% yield was observed, while the ratio of **1b** decreased from 87% to 79% (Table 1). Notably, dehydroalanine **8b** was not detected at −10 °C. At 0 °C, the ratio of **14** increased to 12%, while that of **1b** decreased to 70%, and only 2% of **8b** was detected. These results demonstrate that a Sec−SeOH undergoes an intramolecular cyclization to the cyclic selenenyl amide (Step E), which proceeds faster than the thermal deselenation to the dehydroalanine (Step D).

When cysteine thiol **6** (50 equiv) was added at −20 °C to a solution of **1b** generated in 75% NMR yield, selenenyl sulfide **7b** was formed in a conversion yield of 83% (Scheme 5). In this reaction, the generation of **14** or **8b** was not detected.

The preference among the three possible reaction processes of a Sec−SeOH, i.e., Steps B, D, and E, is summarized as follows: Of the two thermal reaction processes, the intramolecular cyclization (Step E) proceeds faster than the deselenation (Step D). In the presence of a sufficient concentration of thiol, the reaction of a Sec−SeOH with the thiol (Step B) proceeds faster than Steps D and E. These results are consistent with the bypass mechanism as proposed by Flohé et al.[18]

Table 1. Intramolecular Cyclization of 1b to 14



	NMR yield		
temp (°C)	**1b**	**14**	**8b**
−20	87	n.d.	n.d.
−10	79	6	n.d.
0	70	12	2

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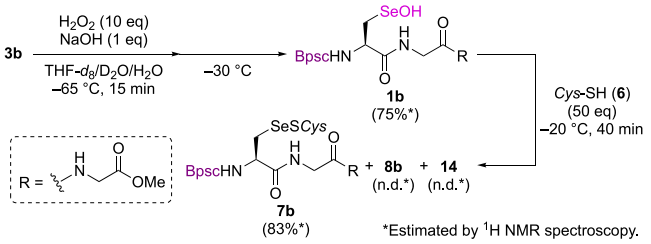
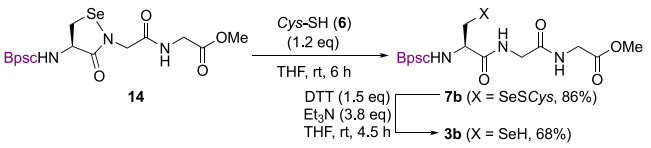
Scheme 5. Reaction of 1b with 6



Cyclic selenenyl amide **14** was converted into selenenyl sulfide **7b** by treatment with thiol **6** in 86% yield (Scheme 6),

Scheme 6. Reaction of 14 with 6 Followed by Reduction with DTT



indicating that the cyclic selenenyl amide form can be reintegrated into the canonical catalytic cycle by reaction with a thiol (Step F). The reduction of **7b** with DTT afforded Sec–SeH **3b**, which corresponds to Step C. Thus, using the Sec-Gly-Gly model, all the chemical processes proposed for the catalytic cycle of GPx, i.e., Steps A–C (Process I) and Steps E and F (Process II), have been experimentally documented in a stepwise manner.

In conclusion, we have presented the first direct observation of selenocysteine selenenic acids (Sec–SeOHs) using cradled selenocysteine and selenopeptide models. The reactivity of the Sec–SeOHs was experimentally examined by ^{1}H and ^{77}Se NMR monitoring, and all chemical processes that have previously been proposed for the catalytic cycle of GPx, including the bypass mechanism, were documented and confirmed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c02383.

Experimental procedures and spectral data (PDF)

Accession Codes

CCDC 2063205 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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(45) To accelerate the rate of oxidation of Sec−SeH, a base was needed.
(46) Sec−SeOH **1a** exhibited a singlet corresponding to the OH proton at 5.35 ppm.
(47) ^{77}Se NMR chemical shifts of previously reported arylmethyl-substituted selenenic acids: **S12** (1243 ppm) and **S13** (1261 ppm); for details, see Table S4.
(48) Cyclic selenenyl amide **14** was also obtained by the intramolecular cyclization of Sec−SeI **5** in the presence of NaOH (Scheme S26). The five-membered ring structure of **14** was determined by 2D NMR spectroscopy (Figure S12). Compound **14** was found to be less susceptible to oxidative deselenation than Sec−SeH **3b** (Scheme S30, Tables S7 and S8).

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