A Model Study on the Effect of an Amino Group on the Antioxidant Activity of Glutathione Peroxidase

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Abstract: In order to investigate mechanistic roles of the amino nitrogens located at the active center of glutathione peroxidase (GPX), a selenium-containing antioxidant enzyme, kinetic analyses and characterization of the intermediates of the model reaction (H_2O_2 + 2PhSH → 2H_2O + PhSSPh) catalyzed by di-2-(*N*-cyclohexyl,*N*-(methylamino)-methyl)phenyl diselenide (**1**) have been performed. The rate equation in methanol at 25 °C by changing the initial concentrations of H_2O_2 and catalyst **1** suggests that the model catalyst (**1**) behaves precisely like GPX in the reduction catalytic cycle. On the basis of the ^{77}Se NMR experiments in a 1:1 mixture of CD_3OD and $CDCl_3$ under rigorous nitrogen atmosphere, three intermediates, selenenyl sulfide **4**, selenolate **5′**, and selenenic acid **6**, are characterized. Three specific roles of proximate nitrogen atoms on the GPX-like activity are proposed. (1) Both theory (MO calculation) and experiments (^{77}Se NMR) suggest that the proximate nitrogen base activates the selenol intermediate (**5**) into the corresponding selenolate anion (**5′**, ^{77}Se NMR observed at δ 22 ppm), which should play a key role in accelerating the catalytic cycle. (2) The proximate nitrogen moiety stabilizes otherwise elusive selenenic acid intermediate (**6**), which is observable by ^{77}Se NMR (δ 1173 ppm). It is suggested by this direct observation of the selenenic acid intermediate that intramolecular Se–N interaction prevents its deterioration by further oxidation in the catalytic system. (3) Since the formation of an Se–N hypervalent bonding is demonstrated by low-temperature dynamic ^{1}H NMR experiment for the selenenyl sulfide intermediate (**4**), it is expected that the nucleophilic attack of benzenethiol (PhSH) should occur preferentially at the sulfur atom of **4**, allowing effective production of the selenolate intermediate (**5′**) in the catalytic cycle.

Introduction

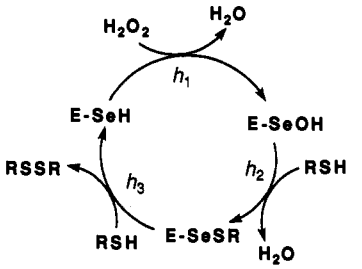
Glutathione peroxidase (GPX),[1] an essential selenium-containing antioxidant enzyme, protects various living organisms from aerobic oxidative stresses by catalyzing the reduction of the metabolites of superoxide anion, such as hydrogen peroxide or lipid hydroperoxides, in the presence of glutathione. The molecular structure of the enzyme determined by X-ray diffraction method[2] has a unique structural feature: two amino groups of glutamine-70 and tryptophan-148 are located at a distance of 3.3 and 3.4 Å, respectively, from the selenium atom of selenocysteine-35 as the active center. Although the importance of these amino residues in the catalytic processes seems to have been recognized in a few recent reports,[3,4] the specific roles of the nonbonded interaction between selenium and the amino nitrogens on the catalytic cycle involving GPX have not been investigated to date. In this paper we present possible mechanistic roles of these amino groups on the GPX enzyme activity based on kinetic analysis and ^{77}Se NMR spectroscopic characterization of three intermediates in a model GPX catalytic reduction of hydrogen peroxide (H_2O_2) (eq 1) by using diselenide **1** as an enzyme model.

$$H_2O_2 + 2PhSH \xrightarrow{\text{catalyst}} 2H_2O + PhSSPh \qquad (1)$$

Results and Discussion

Selection of GPX Model. Although several GPX model compounds have been reported in the literature,[3,5,6] their mechanistic roles in the catalytic reduction of hydrogen peroxide (H_2O_2) are all significantly or slightly different from those in the actual GPX catalytic cycle (Scheme 1).[1] Wilson et al. have reported the interesting result that diselenide **2** and **3**, each of which possesses a basic amino nitrogen near the selenium, exhibit strong antioxidant activity in the GPX assay.[3b] Although the proposed catalytic mechanism, which involves six active intermediates and two reaction cycles, is significantly different from that of GPX, the obvious enhancement of GPX-like antioxidant activity, observed by introducing an amino nitrogen near the divalent selenium, seems strongly indicative of the importance of the amino groups located in the vicinity of the active center of GPX. Recently Hilvert et al. have reported another interesting observation that an artificial enzyme selenosubtilisin, possessing a strongly basic histidine residue at the active center like Wilson's catalyst, exhibits high GPX activity.[4] They concluded that the protonated histidine acts as a general acid facilitating the reduction of the selenenyl sulfide to selenolate with thiols. The presence of a basic amino group therefore seems indispensable as a GPX model compound.

Scheme 1



(E = enzyme, RSH = glutathione)

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(1) Flohé, L. Glutathione Peroxidase Brought into Focus. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: 1982.

(2) Epp, O.; Ladenstein, R.; Wendel, A. *Eur. J. Biochem.* **1983**, *133*, 51.

(3) (a) Reich, H. J.; Jasperse, C. P. *J. Am. Chem. Soc.* **1987**, *109*, 5549. (b) Wilson, S. R.; Zucker, P. A.; Huang, R.-R. C.; Spector, A. *J. Am. Chem. Soc.* **1989**, *111*, 5936. (c) Engman, L.; Stern, D.; Cotgreave, I. A.; Andersson, C. M. *J. Am. Chem. Soc.* **1992**, *114*, 9737.

(4) (a) House, K. L.; Dunlap, R. B.; Odom, J. D.; Wu, Z-P.; Hilvert, D. *J. Am. Chem. Soc.* **1992**, *114*, 8573. (b) House, K. L.; Garber, A. P.; Dunlap, R. B.; Odom, J. D.; Hilvert, D. *Biochem.* **1993**, *32*, 3468.

(5) (a) Müller, A.; Cadenas, E.; Graf, P.; Sies, H. *Biochem. Pharmacol.* **1984**, *33*, 3235. (b) Wendel, A.; Fausel, M.; Safayhi, H.; Tiegs, G.; Otter, R. *Biochem. Pharmacol.* **1984**, *33*, 3241. (c) Parnham, M. J.; Kindt, S. *Biochem. Pharmacol.* **1984**, *33*, 3247.

(6) (a) Fischer, H.; Dereu, N. *Bull. Soc. Chim. Belg.* **1987**, *96*, 757. (b) Haenen, G. R. M. M.; De Rooij, B. M.; Vermeulen, N. P. E.; Bast, A. *Mol. Pharmacol.* **1990**, *37*, 412.

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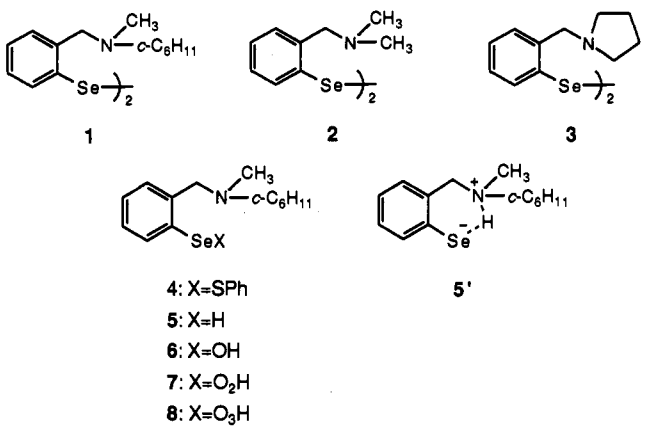


1 2 3

4: X=SPh
5: X=H
6: X=OH
7: X=O_2H
8: X=O_3H

5'

The compound we have chosen as the GPX model is the diselenide carrying a diastereotopic benzylic methylene due to an unsymmetrical tertiary amino group (di-2-(*N*-cyclohexyl,*N*-(methylamino)methyl)phenyl diselenide, **1**), which belongs to a class of Wilson's catalyst:[3b] each nitrogen atom of this compound can interact directly with the nearest selenium atom by forming a five-membered ring as indeed revealed by X-ray analysis of crystalline **1**.[7]

Estimation of GPX-like Activity. Catalytic activity of **1** as a GPX model enzyme was demonstrated according to modified Engman's direct method[3c] using benzenethiol (PhSH) as a glutathione alternative (eq 1). The initial reduction rates of H_2O_2 (v_0), which was obtained by monitoring the UV absorption increase at 305 nm due to diphenyl disulfide (PhSSPh) formed, are listed in Table 1 (entries **a** and **b**) together with those involving other catalysts for comparison (entries **c–h**).

In the absence of catalyst, exceedingly slow oxidation of PhSH took place (entry **c** $v_0 = 0.57\ \mu M \cdot min^{-1}$). While only marginal accelerating effect was observed in the presence of diphenyl diselenide (PhSeSePh) alone (entry **d** $v_0 = 0.67\ \mu M \cdot min^{-1}$), addition of triethylamine (0.05 mM) appreciably enhanced the reduction rate (entry **e** $v_0 = 2.3\ \mu M \cdot min^{-1}$). It should be noted that triethylamine itself was effective as the catalyst (entry **f** $v_0 = 1.7\ \mu M \cdot min^{-1}$), suggesting that nitrogen base plays an important role in the catalytic cycle. In fact, diselenide **1**, having intramolecular tertiary amino groups, significantly accelerated the reaction (entries **a** $v_0 = 34.8\ \mu M \cdot min^{-1}$ and **b** $v_0 = 43.8\ \mu M \cdot min^{-1}$). Similarly, Wilson's catalysts (**2** and **3**)[3b] also showed a high efficiency in our GPX model catalytic system (entries **g** $v_0 = 18.7\ \mu M \cdot min^{-1}$ and **h** $v_0 = 31.2\ \mu M \cdot min^{-1}$).

The above kinetic results suggest two important conclusions as to the effects of an intramolecular amino moiety in a selenium catalyst: (1) The relative v_0 enhancement of PhSeSePh (entry **d**) and NEt_3 (entry **f**) with respect to the uncatalyzed case (entry **c**) is +0.10 and +1.13 $\mu M \cdot min^{-1}$, respectively. Since relative v_0 enhancement of the mixed case (+1.73 $\mu M \cdot min^{-1}$; entry **e**) is much larger than the sum of those in the separated cases (+1.23 $\mu M \cdot min^{-1}$), remarkable cooperative effects of selenium and the basic amino nitrogen on reduction rates is clearly suggested. (2) The significant v_0 enhancement of the selenium catalysts having intramolecular amino nitrogens (entries **a**, **b**, **g**, and **h**) with respect to entry **e** (the intermolecular case) strongly suggests that the selenium as an active center catalyzes the reduction of H_2O_2 by interacting with the proximate basic amino nitrogen.

Rate Equation of the Model Reaction. In order to compare the catalytic cycle of the model system with that of actual GPX, the form of the rate equation of the model reaction (eq 1) was determined using **1**, which exhibited the highest GPX-like activity in the above model assay, as a selenium catalyst.

Lineweaver–Burk plots ($1/v$ vs $1/[PhSH]$ plots) gave linear correlations for various concentrations of the catalyst ([**1**] = 0.001–0.01 mM) (Figure 1). A plot of the line slopes against $1/2[\mathbf{1}]$ gave a linear correlation with the slope of 0.244 ± 0.016 mM·min (Figure 2). Similarly, $1/v$ vs $1/[PhSH]$ plots at various concentrations of H_2O_2 ($[H_2O_2] = 2$–30 mM) (Figure 3) and the subsequent plot, the obtained vertical intercepts vs $1/[H_2O_2]$ plot (Figure 4), gave a linear correlation with the slope and the vertical intercept equal to 15.6 ± 1.1 min and 8.91 ± 0.41 $mM^{-1} \cdot min$, respectively. The complete rate equation of the model

(7) Iwaoka, M.; Tomoda, S. *Phosph. Sulf. Silic. Related Elements* **1992**, *67*, 125.

Table 1. Initial Reduction Rates (v_0)[a] of H_2O_2 (2 mM) with PhSH (1 mM) in the Presence of Various Selenium Catalysts (0.01 mM) in Methanol at 25 °C

entry	catalyst	v_0,[b] $\mu M \cdot min^{-1}$
a	**1**	34.8 (0.6)
b[c]	**1**	43.4 (3.0)
c	none	0.57 (0.07)
d	PhSeSePh	0.67 (0.13)
e	PhSeSePh + NEt_3[d]	2.3 (0.2)
f	NEt_3[d]	1.7 (0.1)
g[c]	**2**	18.7 (0.5)
h[c]	**3**	31.2 (0.6)

[a] Obtained by $1/v$ vs $1/[PhSH]$ plots. [b] Standard deviations are shown in parentheses. [c] The concentration of H_2O_2 was 5 mM. [d] The concentration of NEt_3 was 0.05 mM.

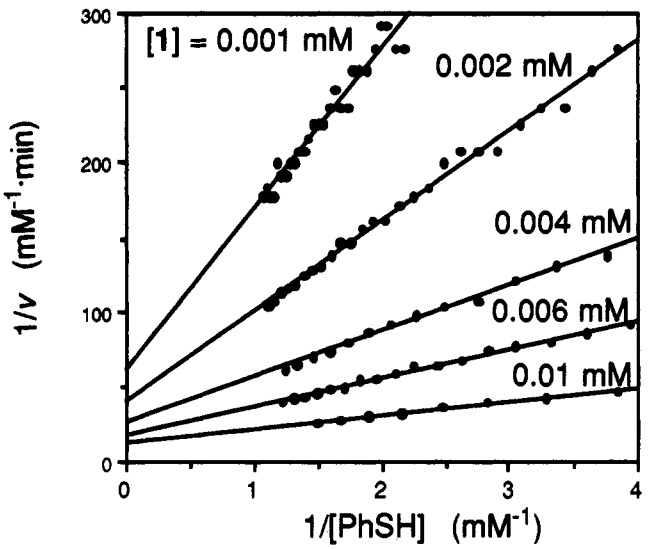


Figure 1. Lineweaver–Burk plot ($1/v$ vs $1/[PhSH]$ plot) of the model reaction (eq 1) changing the catalyst concentration of **1**. The initial H_2O_2 concentration was fixed to 5 mM.

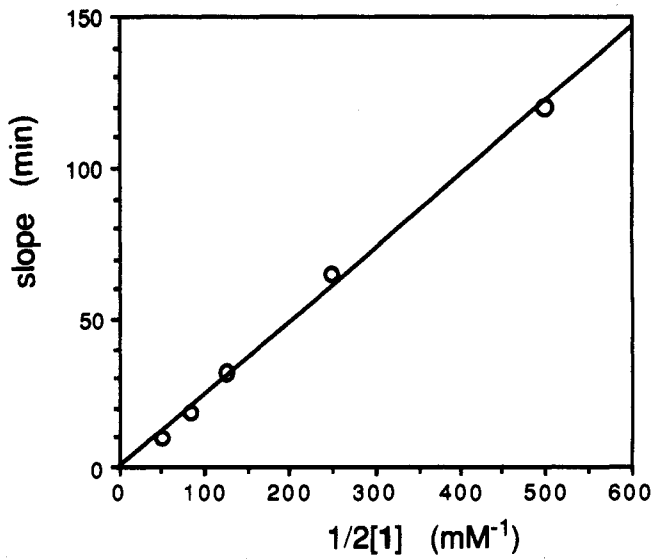


Figure 2. The slope obtained from Lineweaver–Burk plot (Figure 1) vs $1/2[\mathbf{1}]$ plot.

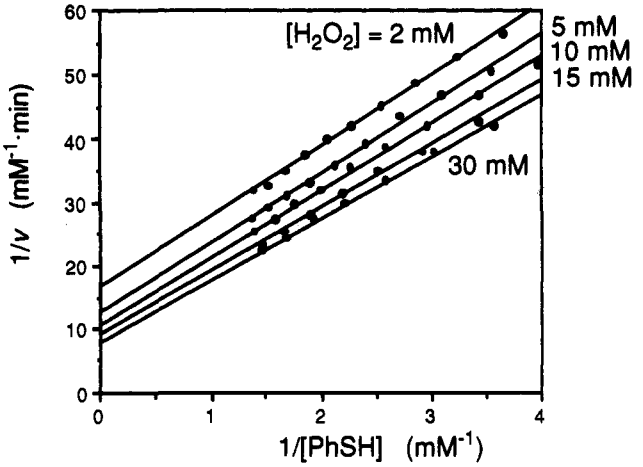


Figure 3. Lineweaver–Burk plot ($1/v$ vs $1/[PhSH]$ plot) of the model reaction (eq 1) changing the H_2O_2 concentration. The catalyst concentration of **1** was fixed to 0.01 mM.

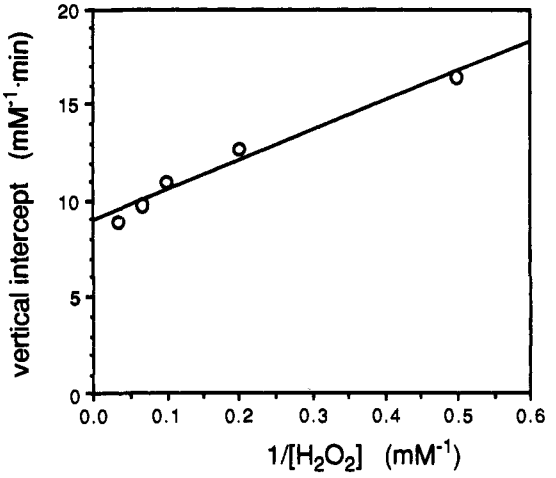


Figure 4. The vertical intercept obtained from Lineweaver–Burk plot (Figure 3) vs $1/[H_2O_2]$ plot.

reaction can therefore be described as eq 2, where k_1 (= 3.2 ± 0.3 $mM^{-1}\cdot min^{-1}$) and k_{23} (= 4.1 ± 0.2 mM^{-1}) mean an apparent bimolecular oxidation process with H_2O_2 and an apparent bimolecular reduction process with PhSH, respectively. The k_4 (= 5.6 ± 0.4 min^{-1}) suggests existence of an apparent unimolecular process presumably due to the formation of catalyst-substrate complexes during the catalytic cycle.[8]

$$1/v = 1/(2[1])\{1/(k_1[H_2O_2]) + 1/(k_{23}[PhSH]) + 1/k_4\} \quad (2)$$

The form of the rate equation thus obtained is identical with that of GPX *in vivo* except for the presence of the $1/k_4$ term, which is negligible in the actual GPX cycle. According to the actual GPX cycle shown in Scheme 1, one can easily obtain the rate equation described as eq 3,

$$1/v = 1/[E]_0\{1/(h_1[H_2O_2]) + 1/(h_2[RSH]) + 1/(h_3[RSH])\} \quad (3)$$

where $[E]_0$ means a total concentration of GPX and h_i means the corresponding second-order rate constant of each process involved in the cycle as indicated in Scheme 1. If h_{23} is defined as $(1/h_2 + 1/h_3)^{-1}$, one obtains the equation like eq 2 except for the presence of the $1/k_4$ term. In the actual GPX cycle, the h_1 value (1.1×10^7 $mM^{-1}\cdot min^{-1}$) is roughly two orders of magnitude larger than the h_{23} value, indicating high antioxidant activity of the enzyme. Although the values of the rate constants (k_1 and k_{23}) obtained in our model system are not so large, similarity of the rate equation between the model and the enzyme clearly suggests that our GPX model compound (**1**) may exactly follow the actual GPX cycle.

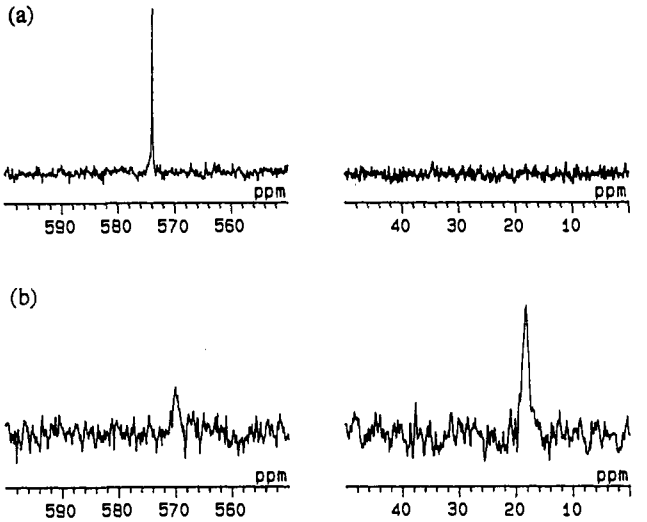


Figure 5. ^{77}Se NMR spectra in 1:1 CD_3OD–$CDCl_3$ under nitrogen atmosphere: (a) isolated **4** and (b) a 1:1 mixture of **4** and PhSH.

Characterization of Intermediates. Having established the basic mechanistic analogy between the model and the actual GPX catalytic cycles, we subsequently proceeded to characterize the intermediates involved in the model catalytic cycle.

Since the reaction between **1** and H_2O_2 was found to be extremely slow as revealed by UV and ^{77}Se NMR spectral changes, the initial step was expected to be the fast reaction of **1** with PhSH. When **1** was treated with an equimolar amount of PhSH in a 1:1 mixture of CD_3OD and $CDCl_3$ under nitrogen atmosphere, two products, the ^{77}Se NMR signals of which appeared at δ 574 ppm and δ 22 ppm with almost equal intensity, were obtained. These signals were unambiguously assigned to those of selenenyl sulfide **4** and selenol **5**, respectively, due to the following reasons. Selenenyl sulfide **4**, isolated as a pure form by column chromatography, was fully characterized by ^1H, ^{13}C, and ^{77}Se NMR (Figure 5a) spectroscopy. On the other hand, the same reactive species as selenol **5** was observed in the reaction mixture of diselenide **1** and sodium borohydride under similar NMR conditions. Thus the initial step was revealed to be the reaction between **1** and PhSH to produce **4** and **5**, both of which should be active intermediates in the catalytic cycle.

The signal of **4** shifted downfield by 48 ppm from that of phenylselenenyl phenyl sulfide (δ 526.0 ppm),[9] strongly suggesting the existence of hypervalent nonbonded interaction between the selenium and the amino nitrogen in **4**.[10] Indeed the 90-MHz NMR absorption of two benzylic protons, which showed up as a singlet at room temperature, coalesced into an AB quartet at 217 K upon cooling. The dynamic NMR spectral behavior of **4** indicates a Se–N interaction of an intermediate strength in **4**.[11] On the other hand, a 123-ppm upfield shift of **5** compared with the chemical shift of benzeneselenol (δ 145 ppm)[12] suggests the presence of significant negative charge on the selenium atom of **5**. When the $CDCl_3$ was used instead of the mixed solvent, the ^{77}Se NMR signal of **5** (observed at δ 47 ppm) was slightly downfield shifted. The remarkable solvent effect on the chemical shift strongly supports structure **5′**, a zwitterionic form of **5**.[13]

(8) Flohé, L.; Loschen, G.; Günzler, W. A.; Eichele, E. *Hoppe-Seyler's Z. Physiol. Chem.* **1972**, *353*, 987.
(9) Luthra, N. P.; Dunlap, R. B.; Odom, J. D. *J. Magn. Reson.* **1982**, *46*, 152.
(10) Analogous Se–O interaction causes similar downfield shift of ^{77}Se NMR: Llabrés, G.; Baiwir, M.; Piette, J.-L.; Christiaens, L. *Org. Magn. Reson.* **1981**, *15*, 152.
(11) The strength of the interaction was evaluated to be 73.2 ± 4.5 kJ/mol by variable-temperature ^1H NMR spectra due to the benzylic protons of isolated **4**. Details will be disclosed in due course.
(12) McFarlane, W.; Wood, R. J. *J. Chem. Soc.* (*A*) **1972**, 1397.

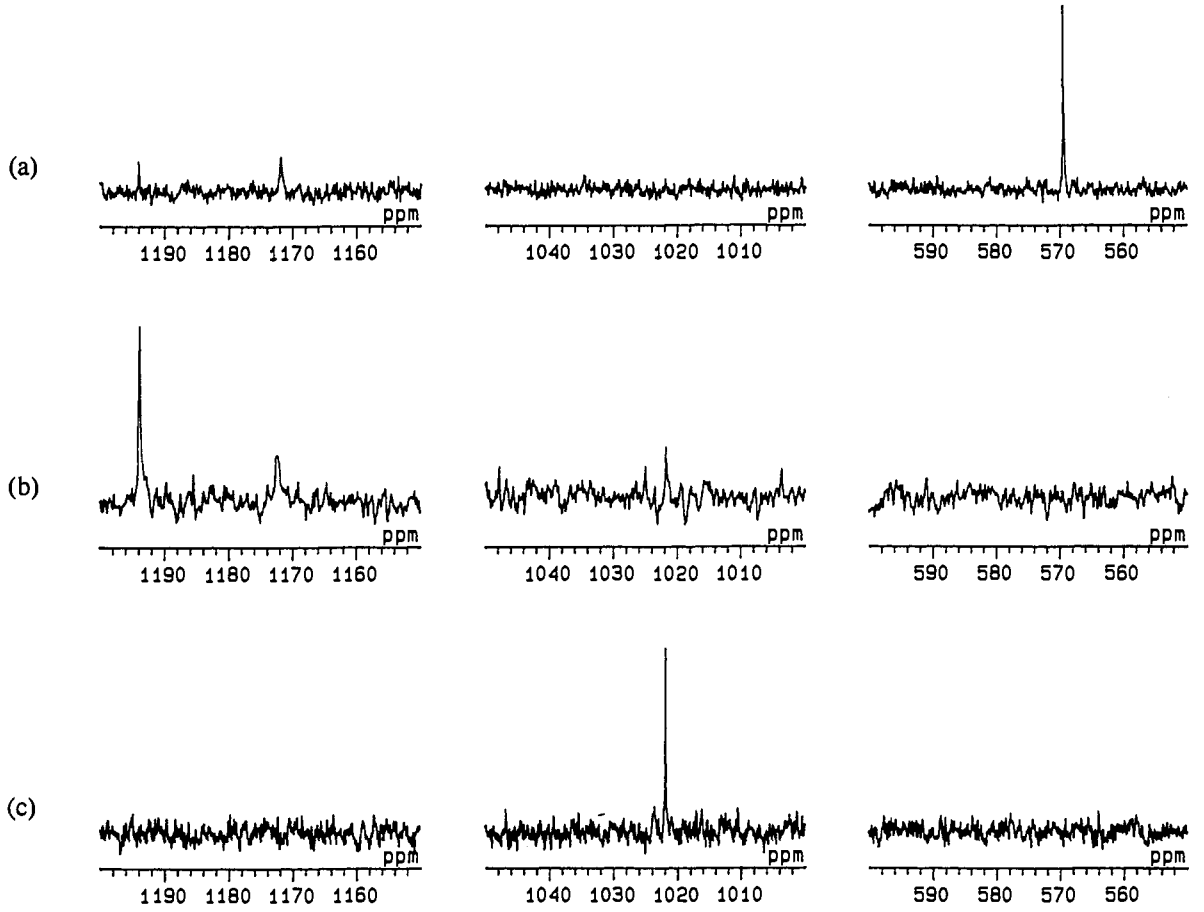


Figure 6. ^{77}Se NMR spectra in 1:1 CD_3OD–$CDCl_3$ under nitrogen atmosphere: (a) a 1:1 mixture of **4** and H_2O_2, (b) a 1:4 mixture of **4** and H_2O_2, and (c) a 1:6 mixture of **4** and H_2O_2.

The existence of the selenolate form is entirely consistent with the results of *ab initio* calculation using 2-(*N*,*N*-(dimethylamino)-methyl)benzeneselenol as a model compound:[14] its fully optimized geometry turned out to be the corresponding zwitterionic form having a hydrogen bond between N^+–H and Se^- as observed in the reduced form of GPX.[2]

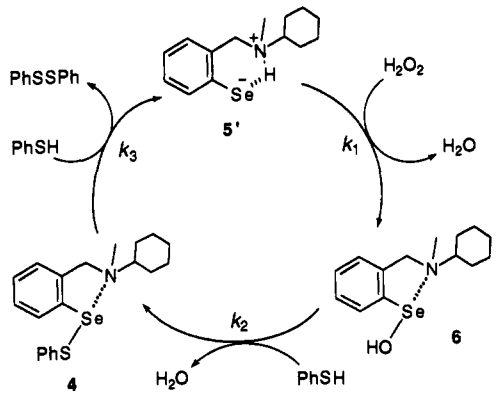
When pure **4** was treated with an equimolar amount of PhSH under identical conditions, single selenium-containing product **5′** was observed by ^{77}Se NMR spectroscopy (Figure 5b), indicating the clean conversion of **4** into **5′** with concomitant formation of diphenyl disulfide (PhSSPh). This reaction should undoubtedly be involved in the catalytic cycle. Importantly when the reaction mixture was treated with an equimolar amount of aqueous H_2O_2, **5′** immediately disappeared to afford a single absorption due to **4** exclusively. This suggests possible intervention of a highly reactive intermediate, such as selenenic acid **6**, as the initial oxidation product of **5′**. That the signal due to diselenide **1** could not be observed during these processes precludes the possibility of its intervention in the catalytic cycle.

Since **6** could not be observed during the oxidation process (**5′** → **4**) presumably due to extremely rapid reaction with PhSH or PhSSPh, direct observation of **6** was undertaken starting from **4** (*i.e.* the reverse of the GPX catalytic cycle). When **4** was treated with an equimolar amount of aqueous H_2O_2 in 1:1 $CDCl_3$–CD_3OD, the ^{77}Se NMR absorption due to **6** was observed at 1173 ppm as a mixture of **4** and seleninic acid **7** (δ 1194 ppm) (Figure 6a). The close resemblance in the chemical shift between **6** and the corresponding acetate (δ 1158 ppm), which was synthesized in spectrally pure form by successive treatment of **1** with equimolar amount of bromine and silver acetate, reasonably supports the assignment of **6**. The possibility that the signal observed at 1173 ppm may be due to the corresponding methyl (CD_3) ester of **6** was excluded because there was no singlet signal around δ 4 ppm due to the methyl (CH_3) protons in the ^1H NMR spectrum measured in 1:1 $CDCl_3$–CH_3OH.[15] Upon further addition of H_2O_2 (4 molar), the relative amount of **7** increased with concomitant formation of selenonic acid **8** (δ 1022 ppm) (Figure 6b), strongly suggesting that the primary oxidation product of **4** with H_2O_2 is **6**. The final product upon further addition of H_2O_2 was **8** (Figure 6c), which was independently synthesized and fully characterized by X-ray diffraction method.[16] The ^{77}Se NMR chemical shift of **6** was considerably downfield shifted compared with that of *o*-nitrobenzeneselenenic acid (δ 1066 ppm)[17] because the selenium atom of **6** strongly interacts with the internal nitrogen atom to form a hypervalent bond as revealed by ^1H NMR of the two diastereotopic benzylic protons which appear as an AB quartet (δ 4.10 and 3.82 ppm, J = 14.7 Hz). This strong interaction may stabilize thermodynamically otherwise labile selenenic acid species involved in the catalytic reduction cycle. It should be noted that all the oxidized products (**6**, **7**, and **8**) rapidly converted into **4** when PhSH was added to the reaction mixture.

Mechanism of the Model Reaction and Possible Roles of Proximate Nitrogen. The above results suggest the following mechanism for the reduction of H_2O_2 with PhSH in the presence of catalyst **1** (Scheme 2). The initial step is the reaction of **1** with

(13) Reich, H. J.; Cohen, M. L. *J. Org. Chem.* **1979**, *44*, 3148.
(14) HONDO version 7.0 was used as a source program. A minimal basis set (STO-3G) was employed for H, C, and N and Huzinaga's 3333/333/3 basis set (*Gaussian basis sets for molecular calculations*; Huzinaga, S., Ed.; Elsevier: 1984) was employed for Se. Details are described in the supplementary material.
(15) The methyl protons of 2-(methoxycarbonyl)benzeneselenenic acid methyl ester resonate at δ 4.07 ppm. Reich, H. J.; Hoeger, C. A.; Willis, W. W., Jr. *J. Am. Chem. Soc.* **1982**, *104*, 2936.
(16) Details are described in the supplementary material.
(17) Reich, H. J.; Willis, W. W.; Wollowitz, S. *Tetrahedron Lett.* **1982**, *23*, 3319.

Scheme 2



PhSH to produce selenenyl sulfide **4** and selenolate **5'**, a zwitterionic form of selenol **5**. Because of hypervalent Se–N interaction, **4** would undergo facile bimolecular displacement preferentially at the sulfur atom with PhSH to give selenolate **5'** (k_3 process), which is highly activated toward oxidation by intramolecular $N^+H\cdots Se^-$ hydrogen bond. Oxidation of **5'** with H_2O_2 would produce selenenic acid **6** (k_1 process), which would then rapidly undergo bimolecular displacement at the selenium atom with PhSH to regenerate **4** (k_2 process). This mechanism is consistent with the rate equation determined by kinetic analysis (eq 2) provided that k_{23} is defined as $(1/k_2 + 1/k_3)^{-1}$.

On the basis of the mechanistic analogy of our model to actual GPX, possible roles of the amino groups present in the proximity of GPX active center may be summarized as follows: (1) The proximate nitrogen base activates the selenol intermediate (E–SeH) into the kinetically much more reactive selenolate anion (E–Se^-). (2) The direct Se–N interaction in the selenenic acid intermediate (E–SeOH) effectively prevents its further oxidation into other oxidized selenium species.[2] (3) The Se–N interaction in the selenenyl sulfide intermediate (E–SeSG) should preclude the nucleophilic attack of a thiol substrate at the selenium, allowing effective regeneration of the selenol intermediate. Although other roles of the proximate amino groups at the GPX active center may be conceivable, the information obtained in this study should be useful for the design and synthesis of highly active enzyme models having GPX-like activity.

Experimental Section

Diselenide **1**, **2**, and **3** were prepared by the condensaton reaction of 2,2'-diselenobis(benzyl chloride) with the corresponding secondary amine according to the conventional method.[7] Commercially available methanol (MeOH), benzenethiol (PhSH), and triethylamine (NEt_3) were dried over appropriate drying agents and distilled under dry nitrogen atmosphere before use. The concentration of aqueous hydrogen peroxide (30% H_2O_2) was determined by titration with potassium permanganate.

Kinetic Analysis. Catalytic GPX model reaction (eq 1) was initiated by the addition of an excess amount of H_2O_2 (2–30 mM) to a methanol solution of PhSH (1 mM = C_0) containing a selenium catalyst (0.001–0.01 mM) and/or NEt_3 (0.05 mM) at 25 °C and was monitored by UV spectroscopy at 305 nm at least more than five times under the same conditions. The molar extinction coefficient of PhSSPh ($\epsilon_1 = 1.24 \times 10^3$ M^{-1} cm^{-1}) at the wavelength was much larger than that of PhSH (ϵ_2 = 9 M^{-1} cm^{-1}). The concentration of PhSH (C) was therefore calculated from the absorbance (a) according to the following equation: $C = (\epsilon_1 C_0 - 2a)/(\epsilon_1 - 2\epsilon_2) + C_0 - 2a/\epsilon_1$. The initial reduction rate of H_2O_2 (v_0) was then determined by $1/v$ vs $1/C$ plots.

^{77}Se NMR Analysis. Unless the particular conditions were specified in the text, 95.35-MHz ^{77}Se NMR spectrum was measured in 0.03 mmol scale under nitrogen atmosphere on a JEOL α-500 instrument by using a 1:1 mixture of $CDCl_3$ (0.25 mL) and CD_3OD (0.25 mL) as NMR solvent with dimethyl selenide as an external standard.

2-(*N*-Cyclohexyl,*N*-(methylamino)methyl)phenylselenenyl Sulfide (4). Diselenide **1** (563 mg, 1 mmol) was dissolved in CH_2Cl_2 under air and benzenethiol (206 mL, 2 mmol) was added to the solution. After 1 h stirring, the reaction mixture was concentrated under reduced pressure. **4** was obtained in 75% yield (583 mg) as a yellow oil from the residue by column chromatography on silica gel (CH_2Cl_2–hexane as eluent). Spectral data for **4**: ^{1}H NMR in $CDCl_3$ δ 1.2–1.3 (m, 5H), 1.6–1.9 (m, 5H), 2.16 (s, 3H), 2.67 (m, 1H), 3.73 (s, 2H), 7.1–7.2 (m, 6H), 7.5–8.0 (m, 2H); ^{13}C NMR in $CDCl_3$ δ 26.0, 26.2, 28.0, 34.3, 60.2, 62.3, 125.7, 125.8, 127.4, 128.0, 128.6, 128.7, 128.8, 135.1, 138.5, 139.2; ^{77}Se NMR in $CDCl_3$ δ 571.9; mass spectrum m/e 391 (M^+), 282 (base peak).

2-(*N*-Cyclohexyl,*N*-(methylamino)methyl)phenylselenenyl Acetate. 2-(*N*-Cyclohexyl,*N*-(methylamino)methyl)phenylselenenyl bromide, quantitatively prepared from **1** (56 mg, 0.1 mmol) by the literature method,[7] was dissolved in CH_2Cl_2, and silver acetate (33 mg, 0.2 mmol) was added to the solution. After 2 h, gray crystals (AgBr) precipitated was removed by filtration, and the filtrate was concentrated under reduced pressure. 2-(*N*-Cyclohexyl,*N*-(methylamino)methyl)phenylselenenyl acetate was quantitatively obtained as red oily material. Spectral data: ^{1}H NMR in $CDCl_3$ δ 1.3 (m, 5H), 1.9 (m, 5H), 2.09 (s, 3H), 2.53 (s, 3H), 3.1 (m, 1H), 3.77 (d, 1H, J = 13.5 Hz), 4.03 (d, 1H, J = 13.5 Hz), 7.2 (m, 3H), 7.7 (m, 1H); ^{13}C NMR in $CDCl_3$ δ 21.9, 25.3, 25.5, 30.4, 37.4, 62.1, 65.7, 125.4, 126.9, 128.5, 133.6, 137.0, 176.0; ^{77}Se NMR in $CDCl_3$ δ 1158.3; mass spectrum m/e 341 (M^+).

2-(*N*-Cyclohexyl,*N*-(methylamino)methyl)benzeneselenonic Acid (8). Diselenide **1** (563 mg, 1 mmol) was dissolved in ether (10 mL), and the solution was added to 0.5 mL of aqueous hydrogen peroxide (30% H_2O_2) three times at an interval of 1 h. After 1 h stirring, precipitated white crystals were collected by filtration and combined with the residue obtained from the aqueous layer of the filtrate. White crystals thus obtained were recrystallized from water to give pure **8** hemihydrate (512 mg, 75% yield). Spectral data for **8**: ^{1}H NMR in CD_3OD δ 1.2–2.2 (br m, 11H), 2.60 (s, 3H), 3.3 (m, 1H), 4.45 (br, 1H), 4.90 (br, 1H), 7.7 (m, 3H), 8.1 (m, 1H); ^{13}C NMR in CD_3OD δ 26.1 (br), 26.4 (br), 29.2 (br), 35.2, 56.3, 66.6, 129.4, 132.5, 134.8, 136.1, 147.1; ^{77}Se NMR in CD_3OD δ 1022. The molecular structure was determined by X-ray diffraction method. A Rigaku automated four-cycle diffractometer was employed with the Cu K_α radiation monochromatized by graphite. The crystal data obtained is as follows. $C_{14}H_{20}NO_3Se\cdot 0.5H_2O$, M = 339.3, monoclinic, a = 21.596(2) Å, b = 12.463(1) Å, c = 15.799(2) Å, β = 134.46(1)°, U = 3034.9(9) Å, space group $C2/c$, Z = 8, D_c = 1.376 g/cm^3. The structure was solved by the direct method and was refined by the full-matrix least-squares method including hydrogen atoms. R-value was reduced to 0.065 for 1971 nonzero reflections. Details are described in the supplementary material.

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Supplementary Material Available: Results of MO calculation of selenol **5** and X-ray structural data of **8** (12 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.