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Biomimetic Studies on Selenoenzymes: Modeling the Role of Proximal Histidines in Thioredoxin Reductases

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The roles of built-in thiol cofactors and the basic histidine (His) residues in the active site of mammalian thioredoxin reductases (TrxRs) are described with the help of experimental and density functional theory calculations on small-molecule model compounds. The reduction of selenenyl sulfides by thiols in selenoenzymes such as glutathione peroxidase (GPx) and TrxR is crucial for the regeneration of the active site. Experimental as well as theoretical studies were carried out with model selenenyl sulfides to probe their reactivity toward incoming thiols. We have shown that the nucleophilic attack of thiols takes place at the selenium center in the selenenyl sulfides. These thiol exchange reactions would hamper the regeneration of the active species selenol. Therefore, the basic His residues are expected to play crucial roles in the selenenyl sulfide state of TrxR. Our model study with internal amino groups in the selenenyl sulfide state reveals that the basic His residues may play important roles by deprotonating the thiol moiety in the selenenic acid state and by interacting with the sulfur atom in the selenenyl sulfide state to facilitate the nucleophilic attack of thiol at sulfur rather than at selenium, thereby generating the catalytically active species selenol. This model study also suggests that the enzyme may use the internal cysteines as cofactors to overcome the thiol exchange reactions.

Introduction

Selenium, an essential trace element, exerts its biological effect through several selenoenzymes, which include glutathione peroxidase (GPx), iodothyronine deiodinase (ID), and thioredoxin reductase (TrxR).[1] Although these enzymes have selenocysteine (Sec) in their active site, their substrate specificity and cofactor systems are strikingly different. The antioxidant enzyme GPx uses glutathione (GSH), whereas the mammalian TrxR prefers internal cysteines as cofactors (Figures 1 and 2).[1,3] In both catalytic cycles, the reduction of selenenyl sulfides by thiols is crucial for the regeneration of the active site. It is known that basic amino groups in the active site of GPx or similar groups in model compounds interact with selenium to modulate its reactivity.[4] Interestingly, although such interactions have been shown to increase the electrophilic reactivity of selenium,[5] these interactions in some of the intermediates become detrimental to the biological activity. For example, strong Se···N interactions in the selenenyl sulfide intermediate in the GPx cycle enhance a nucleophilic attack of thiol at selenium instead of the desired attack at sulfur, leading to a thiol exchange reaction that would hamper the regeneration of the active site selenol.[6,7] Recently, Brandt and Wessjohann have shown that a catalytic triad between Sec, histidine (His), and glutamate (Glu) is essential to stabilize the selenolate form

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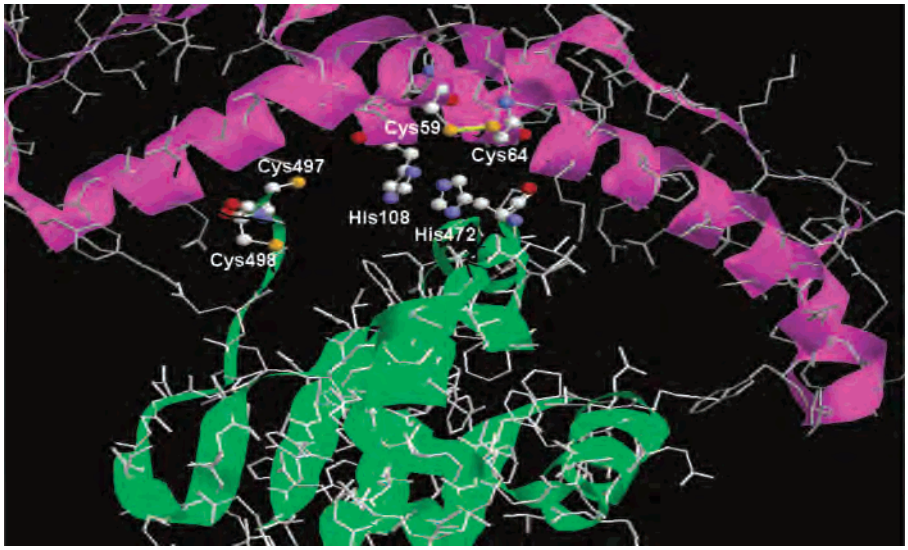

Figure 1. Active site of the SeCys498Cys mutant of rat TrxR (PDB code: 1H6V).[2]

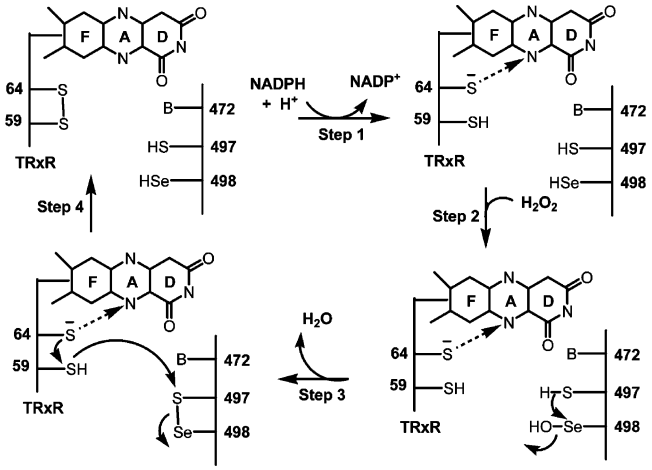

Figure 2. Proposed reaction mechanism for the reduction of H_2O_2 by mammalian TrxR.

of TrxR.[8] On the basis of the X-ray structure of rat TrxR, Brandt and Wessjohann created homology models of human TrxR and subsequently docked to thioredoxin to model the active complex. As a result, the formation of a new type of a catalytic triad between SeCys498, His472, and Glu477 was detected in the protein structure. They have also shown with the help of density functional theory (DFT; B3LYP, lacv3p**) calculations that the formation of such a triad is essential for proton transfer from selenol to His to stabilize a selenolate anion, which can interact with the disulfide of thioredoxin and catalyzes the reductive cleavage of disulfide. Whereas a simple proton transfer from Sec to His is thermodynamically disfavored by ~18 kcal/mol, it becomes favored when the carboxylic acid group of Glu stabilizes the imidazole cation formed during the reaction.

According to the proposed catalytic mechanism,[9] the antioxidant catalytic cycle of TrxR involves three intermediates: a selenol, a putative selenenic acid, and a selenenyl sulfide. In the first step, the selenenyl sulfide receives electrons from nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) via the flavin adenine dinucleotide (FAD) and redox-active dithiol of the first subunit to produce a thiol and a selenol (−SeH) present in the second subunit. Because of the low pK_a value of the selenol, selenolate should be a predominant species under physiological conditions. The selenolate, therefore, is more susceptible to oxidation by H_2O_2 than thiols and reacts with H_2O_2 to form a selenenic acid (−SeOH). At this stage, a cysteine thiol (most likely Cys497) reacts with the selenenic acid to produce water and regenerates the selenenyl sulfide. A second thiol, probably Cys59 from the other subunit, would attack the −Se−S− bridge to regenerate the selenol. Therefore, the selenenyl sulfide serves as either a catalytically essential redox center or transient intermediate during hydroperoxide reduction. In this paper, we describe a model study, for the first time, to show that the more advanced selenoenzymes such as TrxR use proximal His residues for catalysis mainly to overcome the difficulties associated with thiol exchange reactions at the selenium center of the selenenyl sulfide.

Computational Method

All calculations were performed using the *Gaussian98* suite of quantum chemical programs.[10] The hybrid Becke 3−Lee−Yang−Parr (B3LYP) exchange correlation functional was applied for DFT calculations.[11] Geometries were fully optimized at the B3LYP level of theory using the 6-31G(d) basis sets. All stationary points were characterized as minima by corresponding Hessian indices. The NMR calculations were done at the B3LYP/6-311+G(d,p) level on B3LYP/6-31G(d) level optimized geometries using the GIAO method (Figure 7).[12] Orbital interactions and charges

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were calculated using the Natural Bond Orbital (NBO) method at the B3LYP/6-31G(d) level.[13]

Experimental Section

All reactions were carried out under nitrogen or argon using standard vacuum-line techniques. Solvents were purified by standard procedures and were freshly distilled prior to use. ^{1}H (400 MHz), ^{13}C (100.56 MHz), and ^{77}Se (76.29 MHz) NMR spectra were obtained on a Bruker 400-MHz NMR spectrometer. Chemical shifts are cited with respect to $SiMe_4$ (^{1}H and ^{13}C) as the internal standard and Me_2Se (^{77}Se) as the external standard. Mass spectral (MS) studies were carried out on a Q-TOF Micro mass spectrometer with electrospray ionization MS mode analysis. In the case of isotopic patterns, the value given is for the most intense peak. Ebselen,[14] the diselenide **1**,[6a] and the selenenyl iodide **2**[15] were synthesized by following the literature method.

Synthesis of 3. To a stirred solution of selenenyl iodide **2** (25 mg, 0.066 mmol) in CH_2Cl_2 were added triethylamine (9.2 μL, 0.066 mmol) and thiophenol (6.8 μL, 0.066 mmol). After stirring for 1 h at room temperature, the solvent was evaporated under reduced pressure and the product obtained was purified by column chromatography using petroleum ether/ethyl acetate (50:1) as the eluent to give the corresponding selenenyl sulfide as a yellow oil in 92% yield. ^{1}H NMR ($CDCl_3$): δ 8.11 (d, 1H), 7.67 (d, 1H), 7.61 (d, 1H), 7.34−7.45 (m, 5H), 7.11 (t, 2H), 7.02 (t, 1H), 2.75 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 148.9, 137.1, 134.7, 129.1, 128.4, 127.8, 126.2, 125.7, 125.0, 124.7, 124.5, 123.9, 118.1, 46.0. ^{77}Se NMR ($CDCl_3$): δ 624. HRMS (TOF MS ES$^+$): *m*/*z* 360.0346 [(M + H)$^+$].

Synthesis of 4. To a stirred solution of selenenyl iodide **2** (25 mg, 0.066 mmol) in CH_2Cl_2 were added triethylamine (9.2 μL, 0.066 mmol) and *p*-methylbenzenethiol (8.25 mg, 0.066 mmol). After stirring for 1 h at room temperature, the solvent was evaporated under reduced pressure and the product obtained was purified by column chromatography using petroleum ether/ethyl acetate (50:1) as the eluent to give the corresponding selenenyl sulfide in 90% yield. ^{1}H NMR ($CDCl_3$): δ 8.22 (d, 1H), 7.74 (d, 1H), 7.68 (d, 1H), 7.40−7.50 (m, 5H), 7.00 (d, 2H), 2.81 (s, 6H), 2.27 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 148.8, 134.7, 134.5, 133.7, 128.7, 128.4, 128.2, 126.1, 125.6, 124.9, 124.7, 123.9, 118.1, 46.0, 19.9. ^{77}Se NMR ($CDCl_3$): δ 634. HRMS (TOF MS ES$^+$): *m*/*z* 374.0490 [(M + H)$^+$].

Synthesis of 5. To a stirred solution of selenenyl iodide **2** (25 mg, 0.066 mmol) in CH_2Cl_2 were added triethylamine (9.2 μL, 0.066 mmol) and ethanethiol (5 μL, 0.066 mmol). After stirring for 1 h at room temperature, the solvent was evaporated under reduced pressure and the product obtained was purified by column chromatography using petroleum ether/ethyl acetate (20:1) as the eluent to give the corresponding selenenyl sulfide as a colorless oil in 95% yield. ^{1}H NMR ($CDCl_3$): δ 8.32 (d, 1H), 7.68−7.74 (dd, 2H), 7.42−7.51 (m, 3H), 2.79−2.84 (q, 2H), 2.77 (s, 6H), 1.34 (t, 3H). ^{13}C NMR ($CDCl_3$): δ 149.3, 134.8, 129.1, 128.5, 125.7, 124.8, 124.5, 123.6, 118.1. ^{77}Se NMR ($CDCl_3$): δ 569. HRMS (TOF MS ES$^+$): *m*/*z* 312.0315 [(M + H)$^+$].

Synthesis of 7. A stirred solution of 1-(dimethylamino)naphthalene (1.04 g, 6.07 mmol) in dry ether (50 mL) was treated dropwise with a 1.6 M solution of *n*-BuLi in hexane (4.06 mL, 6.50 mmol) via a syringe under nitrogen at room temperature. The mixture was stirred for 24 h, during which time a yellow precipitate of 8-(dimethylamino)-1-naphthyllithium etherate was slowly formed. To this suspension was added rapidly elemental sulfur (0.19 g., 6.08 mmol). After 3 h, all of the sulfur was consumed to produce a yellow solution of lithium naphthylthiolate. To this solution was added 3 M HCl (6.07 mmol), and the reaction mixture was stirred for 1 h. The solvent was then evaporated under reduced pressure, and the product obtained was used as such for further reactions without further purification.

High-Perfomance Liquid Chromatography (HPLC) Assay. In this assay, we employed a mixture containing a 1:1.4 molar ratio of PhSH and H_2O_2 in dichloromethane/methanol (30:70) at room temperature as our model system. Runs with and without 10 mol % of added **3** were carried out under the same conditions. Periodically, aliquots were removed, and the concentrations of the product diphenyl disulfide (PhSSPh) were determined by reverse-phase HPLC, using pure PhSSPh as an external standard.

Results and Discussion

It has already been established that the reduction of selenenyl sulfides by thiols is crucial for the regeneration of the active site in the GPx and TrxR catalytic cycles. Musaev et al. have shown that the reduction of selenenyl sulfides to the corresponding selenols is the rate-limiting step in the catalytic cycle of GPx.[16] Recently, we have also shown that the reduction of the selenenyl sulfide to selenol by incoming thiol is the rate-determining step in the catalytic cycle of ebselen, a cyclic selenium compound that is undergoing clinical trial as an antioxidant.[7] Therefore, we have undertaken a model study on small-molecule selenenyl sulfides having a coordinating amino nitrogen group in the close proximity, to probe the effect of basic His residues in the reactivity of selenenyl sulfides toward nucleophilic attack of a thiol in the TrxR catalytic cycle. It is now well established that ^{77}Se NMR spectroscopy is very sensitive to the electronic environment around the selenium atom and can be used for probing the nature of selenium species in solution. Therefore, the reactivity of the selenenyl sulfides under this present study was followed by using ^{77}Se NMR spectroscopy. It is well-known that the presence of an Se···N interaction in organoselenium compounds leads to a downfield shift of the ^{77}Se NMR signal.[4] Recently, we have shown that the presence of an S···N interaction in selenenyl sulfides leads to an upfield shift of the ^{77}Se NMR signal.[7] To understand the reactivity of selenenyl sulfides toward nucleophilic attack of an incoming thiol, we have synthesized selenenyl sulfides **3**−**6** (Chart 1). The compounds **3**−**5** were prepared from the reaction of bis[8-(dimethylamino)-1-naphthyl]selenenyl iodide (**2**) with PhSH, 4-MePhSH, and C_2H_5SH, respectively. The large downfield shift of the ^{77}Se NMR chemical signals for **3** (624 ppm), **4** (634 ppm), and **5** (569 ppm) strongly suggests the presence of a strong intramolecular nonbonded Se···N interaction in these selenenyl sulfides. On the other hand, the ^{77}Se NMR chemical shift (415 ppm) for the selenenyl sulfide **6**, which was prepared by the addition of the thiol **7** to PhSeSePh, was

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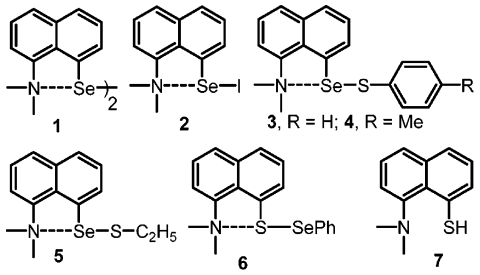
Chart 1



found to be very upfield shifted, suggesting the presence of a strong intramolecular nonbonded S···N interaction.

To find out the effect of the nonbonded Se···N and S···N interactions on the reactivity of selenenyl sulfides toward an incoming thiol, we have carried out several crossover experiments using **3**−**5**, and the reactions were monitored by ^{77}Se NMR spectroscopy and reverse-phase HPLC (Figure 3). The reaction of **3** with *p*-methylbenzenethiol did not

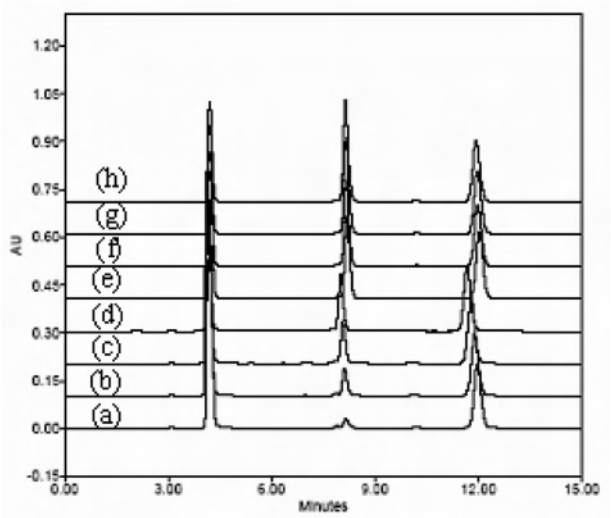


Figure 3. Reverse-phase HPLC chromatogram (254 nm) of **3** (0.7 mM) with PhSH (6.9 mM) and H_2O_2 (9.7 mM). The chromatographic runs were carried out at (a) 0 min, (b) 30 min, (c) 60 min, (d) 90 min, (e) 150 min, (f) 180 min, (g) 215 min, and (h) 245 min.

produce any signal for the selenol even under an inert atmosphere, but this reaction produced a new signal at 634 ppm for a new selenenyl sulfide **4** (Figure S4 in the Supporting Information). Similarly, when **5** was treated with benzenethiol, it did not produce any signal for the selenol, but it produced a new signal at 624 ppm for the selenenyl sulfide **3** (Figure S5 in the Supporting Information). The thiol exchange reactions that take place at the selenium center of **3** and **5** have also been confirmed by reverse-phase HPLC experiments. The reaction of *p*-methylbenzenethiol with the selenenyl sulfide **3** (Figure S8 in the Supporting Information) clearly shows the formation of a new selenenyl sulfide. Similarly, the reaction of benzenethiol with selenenyl sulfide **5** shows the formation of the new selenenyl sulfide **3** (Figure S9 in the Supporting Information). In accordance with the ^{77}Se NMR spectroscopic studies, HPLC chromatograms show no peak corresponding to the selenol or diselenide. These observations strongly suggest that strong Se···N interactions

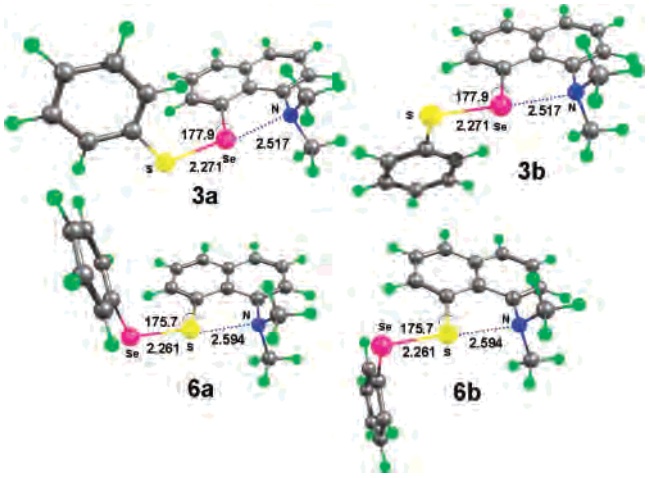


Figure 4. Two possible conformers for selenenyl sulfide **3** and **6**: (a) exo conformer; (b) endo conformer.

in the selenenyl sulfide state lead to thiol exchange reactions, which hamper the regeneration of the active species selenol. Further, we have calculated the rate of reduction of H_2O_2 by PhSH in the presence of **3**. The rate of formation of PhSSPh was monitored by using HPLC at 254 nm. The rate of reaction with **3** (8.51 μM/min) was found to be only 3 times higher than the control rate (2.44 μM/min). The slightly higher rate of reaction could be ascribed to the oxidation of the selenol moiety by peroxide, which might be the driving force behind the reduction of the selenenyl sulfides to selenol. However, the selenenyl sulfide was found to be the major species in the solution even at higher concentrations of thiol, which indicates that the thiol exchange reaction takes place even in the presence of H_2O_2.

The DFT calculations also show that the ^{77}Se NMR chemical shift (573 ppm) of **3** is much downfield shifted compared to that of **6** (424 ppm), indicating the presence of an Se···N interaction in **3** and an S···N interaction in **6**. For model compounds **3** and **5**, two conformers (exo and endo) were found (Figure 4) having almost identical energies. This is in accordance with the report of Tomoda et al. for such compounds with Se···halogen interactions.[17] One conformer has the S/Se−Ph group tilted inward with respect to the naphthalene ring plane (endo), and the other one has the S/Se−Ph group tilted outward (exo). However, only the exo conformer was used for our further studies. The calculations show that the nitrogen atom in **6** interacts with the sulfur (S···N: 2.59 Å), leading to an elongation of the −S−Se− bond (Se−S: 2.27 Å). The NBO second-order perturbation energy for the S···N interaction ($E_{S\cdots N}$) in **6** is calculated to be 11.35 kcal/mol. The calculated ^{77}Se NMR chemical shift for **6** (424 ppm) also shows a large upfield shift compared to that of **3** (573.0 ppm), confirming the expected decrease in the electrophilic character of selenium. The experimental ^{77}Se NMR results are in agreement with the theoretical results, and the upfield shift in the signal observed for **6** (415 ppm) also suggests that the selenium nucleus in **6** is considerably shielded as a result of the S···N interactions

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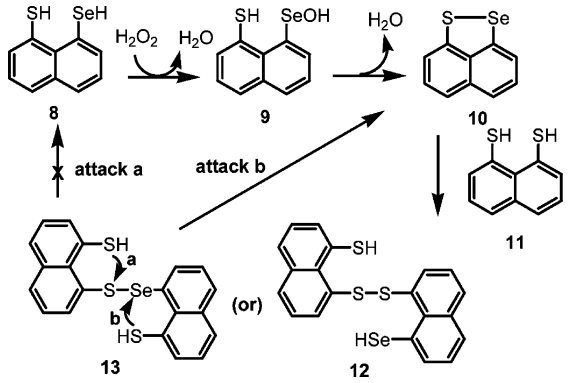

Figure 5. Modeling of the TrxR catalytic cycle.

Table 1. Summary of DFT Calculations and NBO Analysis on **3**−**6** at the B3LYP/6-31G(d) Level[13] and GIAO ^{77}Se NMR Chemical Shifts[12] Calculated at the B3LYP/6-31G(d)//B3LYP/6-311+G(d,p) Level Using the *Gaussian98*[10] Suite of Quantum Chemical Calculations along with Experimental ^{77}Se NMR Chemical Shifts

compd	$r_{S/Se\cdots N}$ (Å)	$E_{Se/S\cdots N}$ (kcal/mol)	q_{Se}	q_S	δ ^{77}Se (calcd)[a] (ppm)	δ ^{77}Se (exptl) (ppm)
3	2.517	18.37	0.301	0.014	573	624
4	2.521	18.14	0.300	0.013	588	634
5	2.548	16.35	0.284	−0.056	673	569
6	2.594	11.35	0.189	0.106	424	415

[a] Referenced to Me_2Se.

even in solution. The NBO analysis shows a decrease in the positive charge on selenium and an increase in the positive charge on sulfur when moving from **3** [0.301 (Se); 0.014 (S)] to **6** [0.189 (Se); 0.106 (S)] (Table 1). This indicates that the nonbonded interactions with sulfur in the selenenyl sulfide intermediate not only increase the electropositive character of sulfur but also decrease the electrophilic character of selenium. In other words, the S···N interactions would certainly enhance the possibility of the thiol attack at sulfur rather than at selenium.

To apply these findings to the mammalian TrxR catalytic cycle, we developed a theoretical model[18] (Figure 5) for the antioxidant properties of the mammalian TrxR. According to this model, the selenol group of **8** reacts with H_2O_2 to form a selenenic acid **9**, which undergoes a further reaction with an internal thiol to form a selenenyl sulfide **10**. The cleavage of the Se−S bond (bond length: 2.259 Å) in **10** by thiol **11** would produce either a disulfide **12** or a selenenyl sulfide **13**, depending upon the position of the thiol attack. The selenium atom in **10** is highly electrophilic, and the natural population analysis (NPA)[13] shows that the selenium atom in **10** carries a more positive charge than the sulfur [0.307 (Se), 0.136 (S)] (Table 2). Recent studies on model compounds show that the nucleophilic attack of thiol at selenium is both kinetically and thermodynamically more favorable than the attack at sulfur.[19] Owing to the electrophilic nature of selenium in **10**, the thiol group of **11** should attack at selenium to produce **13**. The calculations suggest

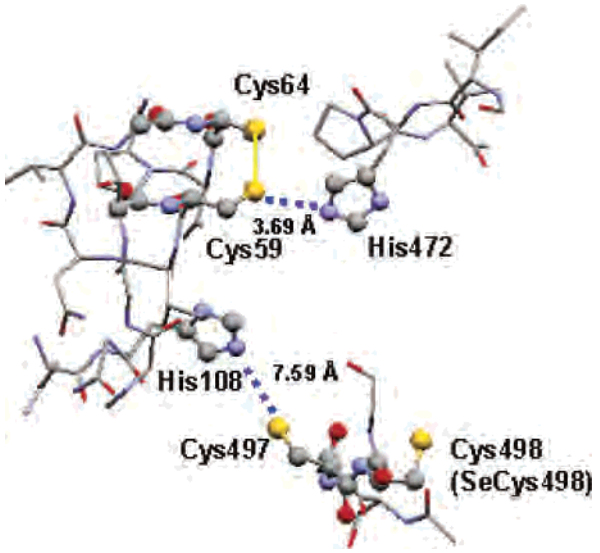

Figure 6. Active site of the SeCys498Cys mutant of rat TrxR showing the close proximity of His472 and His108 to Cys59 and Cys497, respectively (PDB code: 1H6V).[2]

Table 2. Summary of DFT Calculations and NBO Analysis on **10**, **16**, and **18**−**21** at the B3LYP/6-31G(d) Level[13] Using the *Gaussian98*[10] Suite of Quantum Chemical Calculations

compd	$r_{S/Se\cdots N}$ (Å)	$E_{Se/S\cdots N}$ (kcal/mol)	q_{Se}	q_S
10			0.307	0.136
16	2.698	8.70	0.248	0.156
18	2.535	18.02	0.330	0.051
19	2.544	17.67	0.269	0.145
20	2.535	25.29	0.350	0.000
21	2.544	9.40	0.187	0.149

[a] Referenced to Me_2Se.

that compound **13** is more stable than **12** by ∼10 kcal/mol.[18] We propose that the undesired thiol attack b on **13** to regenerate **10** may be preferred over attack a to regenerate the selenol (Figure 5), and this assumption is based on the fact that the selenium atom in compound **13** is more electrophilic than the sulfur atom [0.265 (Se), 0.056 (S)]. In the enzymatic reaction, the cleavage of the Se−S bond by the thiol group of Cys59, followed by reaction with NADPH, effectively regenerates the selenol.[1,20] The attack of Cys59 at sulfur atom of Cys497 is surprising, because the selenium atom in the selenenyl sulfide is expected to be more electrophilic than the sulfur and the formation of an Se−S bond between Cys59 and SeCys498 is expected to occur more readily than the formation of an S−S bond between Cys59 and Cys497.

The reduction of a selenenyl sulfide to selenol in the GPx cycle requires surmounting of a substantial barrier (∼21.5 kcal/mol),[16] and GPx lowers the barrier by bringing the N(Thr54) residue close to the sulfur center of the GSH moiety, which increases the possibility of a nucleophilic attack of negatively charged thiolate at the sulfur atom in

(18) Details of the energy calculations are given in the Supporting Information (Table S1).

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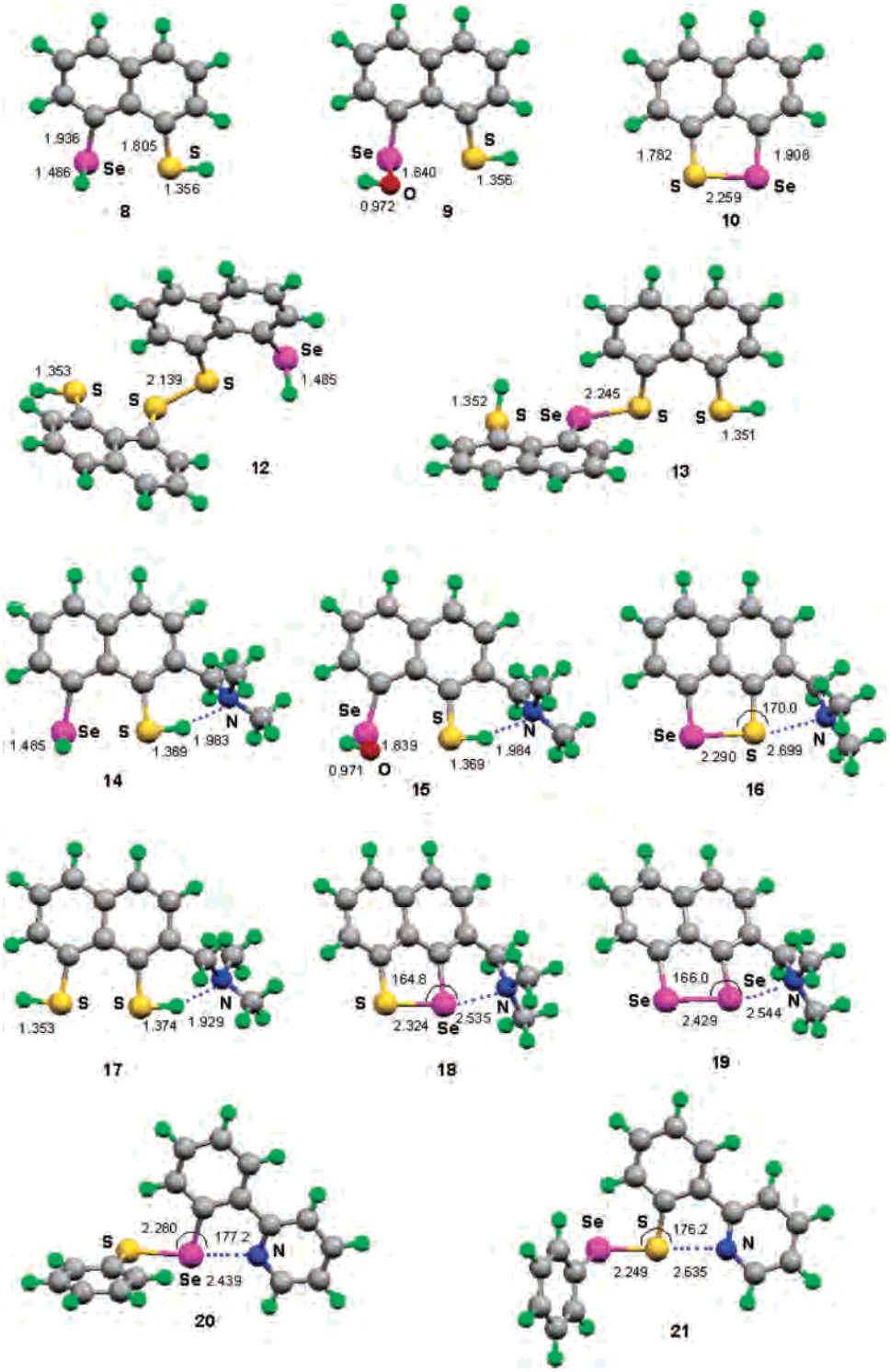
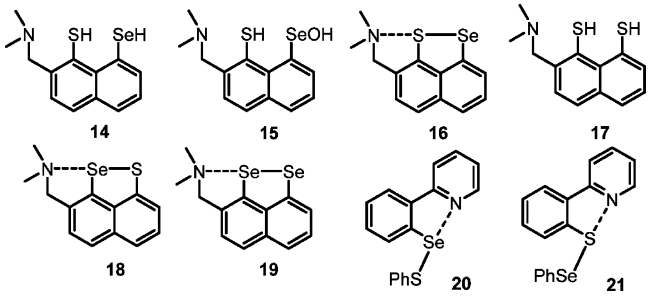


Figure 7. Optimized geometries for **8**−**10** and **12**−**21** obtained by quantum chemical calculations at the B3LYP/6-31G(d) level (only exo conformers are shown).

the −Se−S− bridge.[6b,20] It is not clear whether TrxR uses such a strategy to accomplish the regeneration of the active center. Detailed analysis of the structure of the SeCys498Cys mutant of rat TrxR (Figure 6)[2] reveals that the sulfur atoms of Cys497 and Cys59 are located very close to His108 and His472, respectively. It has been found that the sulfur atom of Cys59 in the −S−S− bridge is at a distance of 3.69 Å from the nitrogen atom of His472 and the sulfur atom of Cys497 is at a distance of 7.59 Å from the nitrogen atom of His108. The conformation of the very C terminal in TrxR can be modeled in such a way that it approaches the redox-active disulfide Cys59−Cys64 close enough for electron transfer without steric clashes, decreasing the distance between Cys59 and Cys497 from 12 to 3 Å. This conformational change involves mainly residues Ser495−Cys498, where the largest movement is that of Cys497 (about a 5-Å displacement of the C_α atom). The charge interaction between the C-terminal carboxyl group of Gly499 and the side chain

Chart 2



of Lys29 can be maintained in the two conformations. In such a conformaional change, the basic His residues can interact with the sulfur atom of the selenenyl sulfide and modulate the reactivity of the selenenyl sulfide.

To understand the role of these basic His residues in catalysis, we carried out DFT calculations at the B3LYP/6-31G(d) level of theory on compounds **14**−**19** (Chart 2), which have internal amino groups. The hydrogen atom bonded to sulfur in **15** is strongly hydrogen-bonded to the nitrogen atom of the amino group (S−H···N: 1.984 Å) to enhance the thiolate attack at selenium. The nitrogen atom in **16** interacts with the sulfur (S···N: 2.698 Å), leading to an elongation of the −S−Se− bond (Se−S: 2.290 Å). NBO analysis[13] at the B3LYP/6-31G(d) level shows the presence of a strong S···N interaction ($E_{S\cdots N}$: 8.70 kcal/mol in Table 2). There is a considerable amount of increase in the positive charge on the sulfur atom and decrease in the positive charge on the selenium atom in **16** [0.248 (Se), 0.156(S)] compared to that of **10** [0.0.307 (Se), 0.136 (S)]. This would certainly enhance the possibility of the thiol **17** attack at sulfur rather than at selenium. As a consequence, the second amino group in the dithiol moiety assists in further reaction of the resulting energetically unfavored disulfide with the internal thiol to regenerate the selenol **14**. The calculated electronic charge shows that the introduction of an amino group in close proximity to the selenium (e.g., **18**) leads to an increase in positive charge in selenium and a decrease in positive charge on the sulfur atom [0.330 (Se), 0.051 (S)], indicating that the Se···N interaction increases the electrophilicity of selenium. Therefore, the thiol is expected to attack at the selenium atom in **18**, which would lead to the formation of a selenenyl sulfide rather than a disulfide. This undesired attack would lead to the regeneration of **18**, as shown in Figure 5 for **13** (attack b).

The NBO analysis shows that there is an increase in the positive charge on selenium and a decrease in the positive charge on sulfur in **18** [0.330 (Se), 0.051 (S)] compared to that of **16** [0.248 (Se), 0.156 (S)]. This would certainly enhance the possibility of the thiol attack at sulfur rather than at selenium in **16**. The increase in the electron density around selenium or the decrease in the electron density around sulfur in **16** by S···N interactions was further verified by replacement of the sulfur atom in **16** with selenium (compound **19**). NPA shows that the selenium atom having intramolecular nonbonded interaction of the amino nitrogen atom carries more positive charge (0.269) than the one having no interaction (0.145).

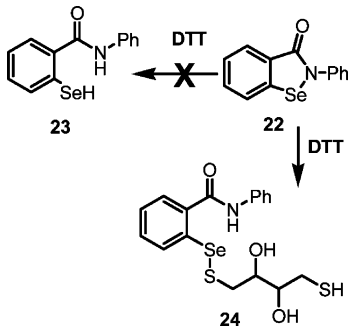


Figure 8. Reaction of ebselen **22** with DTT.

The increase in the electron density around selenium or the decrease in the electron density around sulfur by S···N interactions was further verified by DFT calculations on compounds **20** and **21**, having an aromatic nitrogen atom (pyridine nitrogen) coordinating with selenium and sulfur, respectively (Table 2). As in the case of **3** and **6**, two conformers (exo and endo) were found with negligible differences in energy for compounds **20** and **21**, and the exo conformers were used for further calculations. NBO analysis at the B3LYP/6-31G(d) level indicates that there is a considerable amount of increase in the positive charge on the sulfur atom and decrease in the positive charge on the selenium atom in **21** [0.187 (Se), 0.149(S)] compared to that of **20** [0.0.350 (Se), 0.000 (S)]. This would certainly enhance the possibility of the thiol attack at sulfur in **21**.

We have recently reported the disadvantage of having a coordinating group near the selenium atom in the selenenyl sulfide state.[7] We have also shown that monothiols such as benzenethiol and *p*-methylbenzenethiol do not regenerate the active species selenol from selenenyl sulfides in the GPx cycle of organoselenium compounds as a result of thiol exchange reactions. To investigate the role of the internal cysteines in TrxR, we have carried out reactions by using ebselen (**22**) as a model with 1,4-dithiothreitol (DTT, a dithiol commonly employed as an in vitro cofactor in the ID cycle; Figure 8). The reaction was monitored by ^{77}Se NMR spectroscopy. However, the addition of DTT to ebselen **22** did not produce any signal for the selenol **23**, but it produced the corresponding selenenyl sulfide **24** as a stable product, as revealed by the ^{77}Se NMR spectrum. This study suggests that the use of a dithiol alone is not sufficient for the reduction of selenenyl sulfides to selenols and, as suggested in the GPx cycle, the participation of amino acid residues such as tryptophan or glutamine may be important for the reduction of selenenyl sulfides to selenols. The basic His residues are expected to play similar roles in the catalytic cycle of TrxR.

Conclusion

In summary, these studies on small model selenenyl sulfides show that the mammalian TrxR may use internal cysteines mainly to overcome the thiol exchange reactions and to enhance the reduction of the selenenyl sulfide intermediate. The His residues (His108 and His472) that are very close to the cysteine and Sec residues in the active site may play crucial roles by (i) deprotonating the thiol to

enhance the nucleophilic attack of the thiolate in the selenenic acid intermediate and (ii) interacting with sulfur in the selenenyl sulfide to facilitate a thiol attack at sulfur rather than at selenium. The involvement of two His residues in the catalyses [His108−Cys497−SeCys498] and [His472−Cys59−Cys64] may be responsible for the broader substrate specificity of the mammalian systems as compared with the *Escherichia coli* enzyme.

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Supporting Information Available: Archive entries for the optimized geometries and experimental ^{77}Se NMR spectra of **3**−**5**, **22**, the reaction of ebselen **22** with DTT, the thiol exchange reactions with compounds **3** and **5**, the corresponding HPLC chromatograms, and full reference for ref 10. This material is available free of charge via the Internet at http://pubs.acs.org.

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