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Selenocysteine Confers Resistance to Inactivation by Oxidation in Thioredoxin Reductase: Comparison of Selenium and Sulfur Enzymes

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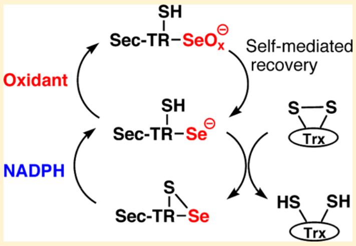
Ⓢ *Supporting Information*



ABSTRACT: Mammalian thioredoxin reductase (TR) is a selenocysteine (Sec)-containing homodimeric pyridine nucleotide oxidoreductase which catalyzes the reduction of oxidized thioredoxin. We have previously demonstrated the full-length mitochondrial mammalian TR (mTR3) enzyme to be resistant to inactivation from exposure to 50 mM H_2O_2. Because a Sec residue oxidizes more rapidly than a cysteine (Cys) residue, it has been previously thought that Sec-containing enzymes are “sensitive to oxidation” compared to Cys-orthologues. Here we show for the first time a direct comparison of the abilities of Sec-containing mTR3 and the Cys-orthologue from *D. melanogaster* (DmTR) to resist inactivation by oxidation from a variety of oxidants including H_2O_2, hydroxyl radical, peroxynitrite, hypochlorous acid, hypobromous acid, and hypothiocyanous acid. The results show that the Sec-containing TR is far superior to the Cys-orthologue TR in resisting inactivation by oxidation. To further test our hypothesis that the use of Sec confers strong resistance to inactivation by oxidation, we constructed a chimeric enzyme in which we replaced the active site Cys nucleophile of DmTR with a Sec residue using semisynthesis. The chimeric Sec-containing enzyme has similar ability to resist inactivation by oxidation as the wild type Sec-containing TR from mouse mitochondria. The use of Sec in the chimeric enzyme “rescued” the enzyme from oxidant-induced inactivation for all of the oxidants tested in this study, in direct contrast to previous understanding. We discuss two possibilities for this rescue effect from inactivation under identical conditions of oxidative stress: (i) Sec resists overoxidation and inactivation, whereas a Cys residue can be permanently overoxidized to the sulfinic acid form, and (ii) Sec protects the body of the enzyme from harmful oxidation by allowing the enzyme to metabolize (turnover) various oxidants much better than a Cys-containing TR.

Selenoenzymes contain the rare and unusual 21st amino acid selenocysteine (Sec, U) in their active sites, and most of these enzymes characterized to date are oxidoreductases.[1,2] Examples of these selenoenzymes are glutathione peroxidase (GPx-1), iodothyronine deiodinase, and thioredoxin reductase (TR). Each of these examples either involves a substrate that is a potent oxidant (hydrogen peroxide/GPx-1), found in an organ whose tissues proliferate due to oxidative stress (thyroid/deiodinase), or is part of an antioxidant system responsible for keeping intracellular protein targets reduced (TR as part of the thioredoxin system).[3−5] A logical hypothesis is that these enzymes evolved to resist inactivation by oxidation.[6,7] This report explores this hypothesis using TR as a test case.

This hypothesis is incongruous with the fact that a selenol (as found in Sec) will oxidize much faster than a thiol.[8] In addition, the selenol/diselenide redox couple has a much lower redox potential in comparison to a thiol/disulfide couple.[9] These chemicophysical properties led early researchers in the field to declare: “...this highly oxidizable amino acid (Sec) could be maintained only in anaerobic organisms or in aerobic systems which evolved specific protective mechanisms.”[10] and that Sec “...is perhaps a vestigial reminder of the anaerobic world of 2 or 3 billion years ago.”.[11] Yet, we find Sec in the active sites of seleno-oxidoreductases that are involved in oxidant metabolism. How can this paradoxical situation be explained?

We have recently introduced the concept of the “selenium paradox” with respect to the use of Sec and oxygen metabolism.[6,7] While Sec is oxidized rapidly in the presence of oxidants such as H_2O_2 due to the high nucleophilic character of a selenolate, the resulting *Se*-oxides of Sec, selenenic acid (Sec-SeOH) and seleninic acid (Sec-SeO_2^-), can be rapidly reduced back to the selenol due to the high electrophilic character of the selenium atom of the oxide. Sulfur in the form of cysteine (Cys) can also be highly nucleophilic and react with oxidants to form analogous *S*-oxides. A key difference between the oxides of the two elements is that the sulfinic acid form of Cys (Cys-SO_2^-) is extremely resistant to chemical reduction by thiols.[12] A Cys-SO_2^- residue can be reduced back to a thiol in the case of peroxiredoxin, but this requires the use of a repair enzyme (sulfiredoxin) to catalyze the conversion back to Cys-SH.[13] Further, cysteine−sulfinic acid can be readily oxidized to the sulfonic acid (Cys-SO_3^-), while Sec-SeO_2^- resists further oxidation to the selenonic acid (Sec-SeO_3^-) form.[6] These

Received: April 13, 2013
Revised: July 17, 2013
Published: July 18, 2013

dx.doi.org/10.1021/bi400462j | *Biochemistry* 2013, 52, 5472−5481

properties of selenium and the "Janus-faced" nature of selenium, high nucleophilicity and high electrophilicity, make this element well suited to metabolize oxidants in enzymes that need to be highly resistant to inactivation by oxidation.[14]

Here we show that mammalian Sec-containing TR strongly resists inactivation by oxidation in comparison to a Cys-orthologue from *D. melanogaster* (DmTR) using a wide range of oxidants including: H_2O_2, hydroxyl radical, peroxynitrite, and hypohalous acids. Our hypothesis was also tested by creating a mutant of DmTR in which the active-site Cys residue is replaced with Sec (referred to in this paper as the "Sec-rescue" enzyme). The resulting mutant becomes very resistant to inactivation by oxidation due to this switch of a single atom in the enzyme. The findings in this report provide the first experimental evidence for the superior ability of a Sec-enzyme to resist oxidative inactivation over its Cys-orthologue tested under identical conditions.

■ MATERIALS AND METHODS

Materials. NADPH was purchased from AppliChem (Darmstadt, Germany). Lactoperoxidase from bovine milk, DTNB, catalase from bovine liver, isoamyl nitrite, and sodium hypochlorite were all obtained from Sigma-Aldrich (St. Louis, MO). Ferrous chloride, hydrogen peroxide (30% solution), potassium thiocyanate, isopropyl alcohol, and EDTA were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium hypobromite solution was obtained from Aqua Solutions (Deer Park, TX). Enzyme kinetic assays were performed on a Cary50 UV−vis spectrophotometer (Walnut Creek, CA). DMPO was obtained from Cayman Chemical Company (Ann Arbor, MI). All kinetic assays were conducted at room temperature unless otherwise noted. For EPR experiments, 100 μL borosilicate glass capillaries were from Kimble Chase (product # 34502-99).

Procedure for the Preparation of Peroxynitrite ($ONOO^-$). Peroxynitrite was synthesized as described by Uppu and colleagues.[15] Briefly, fresh solutions of 50% (v/v) isopropyl alcohol/water and 0.55 M NaOH were prepared. Next, 18 mL of 0.55 M NaOH was mixed with 20 mL of 50% isopropyl alcohol with constant mixing at 25 °C. To this solution was added 0.93 mL of 9.5 M H_2O_2 and 1.11 mL of isoamyl nitrite (sequentially) and allowed to mix for 15 min. The newly synthesized $ONOO^-$ was then washed extensively in a separatory funnel with dichloromethane to remove any residual organic solution. The solution of $ONOO^-$ was then loaded onto a column of manganese dioxide (10 g) to quench any excess remaining H_2O_2 followed by purging with argon for 15 min. The concentration of $ONOO^-$ was determined spectrophotometrically before each experiment via its absorbance maximum at 302 nm (ε = 1670 M^{-1} cm^{-1}).[16]

Procedure for the Preparation of Hypochlorite (HOCl) and Hypobromite (HOBr). Hypochlorite was freshly prepared before each experiment by the dilution of a 10−15% stock solution of NaOCl into a solution of 0.9% NaCl, and its concentration was determined spectrophotometrically by the absorbance peak of ^{-}OCl at 292 nm (ε = 350 M^{-1} cm^{-1}) at a pH range of 10−12.[17] The concentration of HOCl in the resistance assay ranged from 0 to 100 μM. Sodium hypobromite solution was purchased from a commercial supplier (Aqua Solutions, Deer Park, TX), and the concentration of the stock solution was determined spectrophotometrically before each experiment by the absorbance maximum of ^{-}OBr at 329 nm (ε = 332 M^{-1} cm^{-1}) at a pH range of 10−13.[18] Working stocks of 500 μM HOBr were freshly prepared before each individual incubation experiment by dilution into 100 mM sodium phosphate, pH 7.4 buffer. The concentration of HOBr in the resistance assays ranged from 0 to 20 μM.

Procedure for the Preparation of Hypothiocyanous Acid (HOSCN). The enzymatic preparation of HOSCN by lactoperoxidase (LPO) has been previously described.[19] Briefly, LPO from bovine milk, potassium thiocyanate (KSCN), and hydrogen peroxide (H_2O_2) were all freshly prepared in deionized water prior to each experiment. The concentrations of LPO and H_2O_2 were determined spectrophotometrically by their absorbance maxima at 412 nm (ε = 112 000 M^{-1} cm^{-1})[20] and 240 nm (43.6 M^{-1} cm^{-1}), respectively. The enzymatic synthesis of HOSCN was initiated by the reaction of 7.5 mM KSCN and 3.75 mM H_2O_2 (added in five individual aliquots at 1 min intervals to prevent inactivation of LPO) to a 2 μM LPO solution in 10 mM potassium phosphate, pH 6.6 buffer. The 2 mL reaction was allowed to proceed for 15 min at room temperature after which catalase (10 μg/mL) was added to quench any excess H_2O_2 for an additional 5 min. HOSCN was then concentrated by ultrafiltration using Amicon Ultra 30 devices (Millipore, MA) in a microcentrifuge at 11 000 rpm for 5 min at 4 °C. The concentration of HOSCN was quantified by monitoring the loss in TNB anion at 412 nm. Briefly, 1.5 mM DTNB was first treated with 50 mM NaOH to chemically cleave the disulfide, and the initial concentration of TNB anion was determined spectrophotometrically by its absorbance maximum at 412 nm (ε = 14 100 M^{-1} cm^{-1}).[21] Next, 10 μL of freshly synthesized HOSCN was added to the TNB solution and allowed to react for 15 min. The TNB solution was then placed into the spectrophotometer, and the ΔA_{412} (before and after HOSCN addition) was used to calculate the concentration of HOSCN. The concentration of HOSCN in the resistance assays ranged from 0 to 500 μM.

Construction of the Semisynthetic DmTR-SCUG Mutant Enzyme. The production and purification of the recombinant DmTR-SC<u>C</u>S[a] and the semisynthetic mTR3-GC<u>U</u>G enzyme used in this study have been previously reported.[22,23] In order to replace the active site <u>Cys</u> nucleophile of DmTR (Cys490) with Sec, we created a DmTR-intein fusion protein in which the final three amino acids (Cys-Cys-Ser) are missing. A tripeptide of sequence H-Cys-Xaa-Yaa-OH can then be ligated to the enzyme using the technique of protein semisynthesis. We have previously reported on the procedures for construction of this fusion protein and the conditions needed to carry out peptide ligation in order to create mutants of DmTR by semisynthesis.[22] The procedure for the synthesis of peptide H-CUG-OH and its ligation to a thioester-tagged DmTRΔ3 construct was analogous to that previously described in our work with the mammalian mitochondrial semisynthetic enzymes.[23] The concentration of the purified DmTR-SCUG enzyme was determined based on the extinction coefficient of flavin at 460 nm (ε_{460} = 22.6 mM^{-1} cm^{-1} for the dimer).[24]

Activity Assays with Trx and H_2O_2. Activity assays following NADPH consumption were monitored by the decrease in absorbance at 340 nm (A_{340}) using an extinction coefficient of 6220 M^{-1} cm^{-1}.[24] Stock solutions of H_2O_2 were freshly prepared in 50 mM potassium phosphate, pH 7.0 buffer before each experiment with the concentration of H_2O_2 determined spectrophotometrically using an extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm.[25] The conditions of both the Trx- and H_2O_2-reductase assays have been previously described.[26] Briefly, the Trx-reductase assay comprised of a 0.5 mL reaction containing 1 mM EDTA, 0.16 mM bovine pancreas insulin, 150 μM NADPH, and 25 nM DmTR-SCUG enzyme in 50 mM potassium phosphate, pH 7.0 buffer. The concentration of *E. coli* thioredoxin (Trx) varied from 0 to 210 μM. The H_2O_2-peroxidase assay contained 2 mM EDTA, 200 μM NADPH, and either 50 nM DmTR-SCUG or 40 nM DmTR-SCCS in 100 mM potassium phosphate, pH 7.0 buffer. The concentration of H_2O_2 ranged from 0 to 70 mM. Background NADPH consumption was corrected for by subtracting the activity of control experiments in which either TR or substrate (Trx or H_2O_2) was omitted from the reaction. The assays were repeated in triplicate for each enzyme.

Measuring Trx-Reductase Activity of TR Enzymes after Incubation with NADPH and H_2O_2. In order to directly compare the ability of Cys- and Sec-TRs to resist peroxide-induced inactivation, we developed a two-part assay in which we first exposed the reduced TR enzymes to increasing concentrations of H_2O_2 (0−50 mM) and then assessed the remaining Trx-reductase activity relative to a control reaction where H_2O_2 was omitted. Three different TR enzymes were utilized in these studies: the mammalian mitochondrial WT enzyme (mTR3-GCUG; 45 nM), the *Drosophila melanogaster* WT enzyme (DmTR-SCCS; 100 nM), and the DmTR Sec-rescue enzyme (DmTR-SCUG; 45 nM). We first reduced the enzymes with NADPH (200 μM) for 5 min, followed by the addition of increasing amounts of H_2O_2 (0−50 mM) to the 0.7 mL reaction mixture also containing 1 mM EDTA in 100 mM potassium phosphate buffer, pH 7.0. The TR enzymes were incubated with oxidant for 25 min upon which catalase (14 Units) was added to the reaction to quench excess H_2O_2 in an additional 12 min incubation. An additional bolus of 200 μM NADPH followed by 90 μM *E. coli* Trx was next added to the reaction, and the Trx-reductase activity was measured by monitoring the consumption of NADPH as a decrease in A_{340}. The assays were repeated in triplicate for each enzyme. For each assay a "no Trx" control was performed in which the background activity was measured with an equal volume of buffer included in the assay in place of Trx. In each of these control experiments, no activity was detected. Background consumption of NADPH was subtracted from the experimental activity by carrying out a control experiment in which TR was omitted from the reaction. The change in absorbance was converted to specific activity (mol of NADPH min^{-1} mol^{-1} of TR), and the ability to resist oxidant-induced inactivation was quantified as a percentage of activity remaining relative to the oxidant-untreated control. Figure S1 shows plots of absorbance vs time for the hydrogen peroxidase assays as an example of the raw data generated in this work. As described above, this raw data was then converted into an activity and then subsequently plotted as bar graphs as discussed in the Results and Discussion.

Measuring DTNB Reductase Activity after Incubation with NADPH and H_2O_2. Two truncated constructs lacking the C-terminal redox center (DmTRΔ8 and mTRΔ8)[a] were employed to directly test the ability of the N-terminal CVNVGC redox center to resist inactivation by oxidation. The full-length TR enzymes mTR3-GCUG, DmTR-SCCS, and DmTR-SCUG were used to assess how the Sec residue could serve to protect the N-terminal redox center relative to Cys. The enzymes were reduced with 200 μM NADPH in potassium phosphate buffer, pH 7.0, followed by immediate addition of H_2O_2 to a final concentration of 50 mM. Following quenching by catalase, fresh NADPH (200 μM) was added to the 0.7 mL assay along with 0.5 mM DTNB. The DTNB-reductase activity was measured by monitoring an increase in absorbance at 412 nm (A_{412}) corresponding to the formation of TNB^- anion from DTNB. Enzyme concentrations were as follows: mTRΔ8 (10 nM), DmTRΔ8 (45 nM), DmTR-SCCS (45 nM), DmTR-SCUG (45 nM). A lower concentration of mTRΔ8 was utilized due its more robust DTNB reductase activity. The assays were repeated in triplicate for each enzyme.

EPR Experiments with EDTA-Chelated Ferrous Iron and H_2O_2. To confirm the generation of the radical oxidants from the Fenton reaction, we carried out electron paramagnetic resonance (EPR) experiments using the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Oxygen radicals are short-lived species; their generation and identity can be detected and characterized by the unique EPR spectrum that results from the trapping of free radicals by the spin trap.[27−29] Two different reaction conditions were assessed. First, to monitor any radical generation in the absence of H_2O_2 ("Fe-oxidant"), a 20 μM $FeCl_2$−100 μM EDTA complex was mixed with 50 mM DMPO in 50 mM potassium phosphate, pH 7.0 buffer. The reaction mixture was transferred to a 100 μL EPR tube, and the spectra were collected on a 9.2 GHz Bruker X-band EPR spectrometer at the Geisel School of Medicine, Dartmouth University (Lebanon, NH). The second reaction consisted of identical components, except 100 μM H_2O_2 was also included to generate the hydroxyl radical ($^\bullet$OH). The settings on the EPR spectrometer were as follows: the microwave power was set to 6.33 mW, the modulation amplitude was 0.2 G, the scan range was 24.887 G, the scan time was 20.9 s, and the number of scans was set at 5.

Activity Knockdown Assay for $^\bullet$OH and Fe-EDTA Oxidant-Treated Full Length Cys- and Sec-TR Enzymes. To assess the ability of Cys- and Sec-TRs to resist inactivation from exposure to iron generated oxidants in the absence of H_2O_2 and the iron-generated hydroxyl radical in the presence of H_2O_2, we utilized EDTA-chelated ferrous iron and H_2O_2, components of the well-known Fenton reaction. In initial experiments we assessed the effect of iron-generated oxidants (absence of H_2O_2) on TR activity by reacting ferrous chloride (20 μM) with a 5-fold excess of EDTA chelator (100 μM) (5:1 ratio of chelator to iron). The TR enzymes (45 nM) were first incubated with 200 μM NADPH in a 5 min reduction phase. Next, the chelated Fe-EDTA mixture was added to the 0.7 mL reaction also containing 50 mM potassium phosphate, pH 7.0, and incubated for 20 min. In order to quench both the generated oxidant and remaining redox active iron, 1 mM glutathione and 1 mM deferoxamine chelator were added to the reaction and incubated for an additional 10 min, as per published protocols.[30] Next, 90 μM *E. coli* Trx was added to the reaction, and the remaining Trx-reductase activity of the TR enzymes was assessed by following the NADPH consumption via a decrease in A_{340}. In subsequent experiments employing identical reaction conditions, H_2O_2 was added to the reaction in an equimolar ratio to EDTA (100 μM) to generate $^\bullet$OH. We also carried out a control experiment in which only 100 μM H_2O_2 (in the absence of $FeCl_2$) was included in the incubation. The activity profiles of the Fe-EDTA alone, H_2O_2 alone, and Fe-EDTA-H_2O_2 treated TR enzymes were compared to those of individual control experiments omitting both Fe−H_2O_2 and Fe. The assays were repeated in triplicate for each enzyme, and the background activity was subtracted as described above.

General Procedure for the Oxidant Resistance Assays of Cys- and Sec-TRs Treated with $ONOO^-$, HOCl, HOBr, and HOSCN. The reaction conditions and incubation protocol for the oxidant resistance assays with $ONOO^-$, HOCl, HOBr, and HOSCN were all identical. The preparation of each oxidant and the corresponding oxidant concentration ranges employed in each respective experiment have been described above. The enzyme concentrations in each assay were as follows: 100 nM DmTR-SCCS, 45 nM DmTR-SCUG, and 45 nM mTR3-GCUG. The sample TR enzymes were first prereduced with excess NADPH (200 μM) for 5 min in a 0.5 mL reaction mixture also containing 1 mM EDTA in 100 mM potassium phosphate, pH 7.0 buffer. Following the oxidant incubation, an additional bolus of 200 μM NADPH was added to the cuvette followed by 90 μM *E. coli* Trx. The remaining Trx-reductase activity was then assessed by monitoring the consumption of NADPH by a loss in absorbance at 340 nm. The assays were repeated in triplicate for each enzyme, and a "no Trx" control was performed for each assay as described above. Each assay was corrected for background activity as described above.

■ RESULTS AND DISCUSSION

Resistance against Inactivation by H_2O_2. Previously, we reported that the mammalian mitochondrial Sec-containing TR effectively resists inactivation when exposed to H_2O_2 concentrations as high as 50 mM, at both neutral and acidic pH.[31] We attributed this ability to recover from oxidative damage to the increased electrophilicity of a *Se*-oxide relative to a *S*-oxide.[6,7,32] In this report we have directly compared the ability of Cys- and Sec-TR orthologues to recover from inactivation under identical conditions of high oxidant concentration. For the Cys-TR, we used the enzyme from *D. melanogaster*, abbreviated as DmTR-SC<u>C</u>S.[a] This Cys-orthologue is both structurally and evolutionarily related to the mouse mitochondrial TR enzyme, abbreviated as mTR3-GC<u>U</u>G.[26,33] We note that the Cys mutant of mTR (mTR-GCCG) was not employed in these studies due to its inherently low Trx reductase activity, which prevents a readout of resistance from inactivation. We first compared the ability of these Cys- and Sec-TR enzymes to resist inactivation from exposure to H_2O_2 in a concentration range of 1−50 mM. The activity resistance profile shown in Figure 1 demonstrates that the Cys-containing enzyme loses >50% of activity upon exposure to 1 mM H_2O_2 while suffering a more severe activity loss when incubated with higher H_2O_2 concentrations ~15% activity remaining with 50 mM H_2O_2. The results are similar whether or not we performed a peroxide quenching step with catalase. In contrast, the mammalian Sec-TR enzyme is unaffected by treatment with 1−10 mM H_2O_2 and still retains ~75% of activity when incubated with 50 mM H_2O_2.

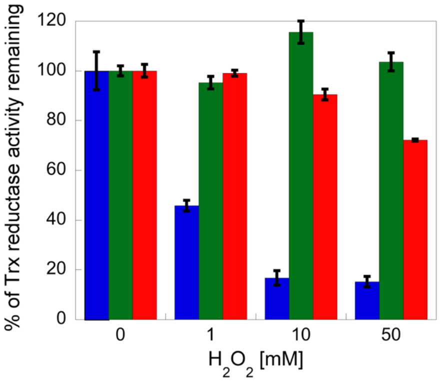


Figure 1. Resistance to inactivation from H_2O_2 by Sec- and Cys-TR enzymes. The prereduced TR enzymes were incubated with increasing concentrations of H_2O_2 (0−50 mM) followed by activity assessment with 90 μM *E. coli* Trx. The blue bars represent the WT Cys-TR from *D. melanogaster* (DmTR-SCCS), the green bars represent the DmTR-SCUG "Sec-rescue" enzyme, and the red bars represent the WT Sec-TR from mouse mitochondria. For this and all similar plots, (i) the activities represent the amount of activity remaining relative to the oxidant-untreated control and (ii) the error bars represent the standard deviation from three trials and are normalized to the percentage of remaining activity.

To further test our hypothesis that a Sec residue confers resistance to inactivation by oxidation, we generated a mutant of the Cys-containing TR, DmTR-SC<u>C</u>S, in which the active site <u>**Cys**</u>-nucleophile was replaced with a Sec residue using semisynthesis. This mutant enzyme is abbreviated as DmTR-SC<u>U</u>G. In effect, it is a chimeric enzyme in which we removed the Cys-containing "tail" of the enzyme and then grafted on a Sec-containing tail to the Cys-enzyme "body". We note that in the construction of this chimeric enzyme a Gly residue was used in place of Ser in order to prevent any protecting group migration from the Se atom of Sec to the adjacent hydroxyl group of Ser during solid phase peptide synthesis. We refer to this enzyme as the "Sec-rescue" enzyme throughout the rest of this study. We subsequently subjected this Sec-rescue enzyme to identical assays as described above to see if this enzyme has now gained the ability to resist inactivation by oxidation using H_2O_2 as the oxidant.

The results with the Sec-rescue enzyme are also shown in Figure 1. These results indicate that the substitution of a single atom (not including the terminal Gly for Ser substitution) in the entire DmTR enzyme renders the enzyme resistant to H_2O_2-induced inactivation. This result is in direct contrast with the original hypothesis that selenium is so sensitive to oxidation that it could only be used in enzymes where it is "protected" from oxidation.

Resistance against Inactivation by One-Electron Oxidants. Previously, Koppenol and colleagues predicted Sec-enzymes may better resist damage from one-electron oxidants then their Cys-orthologues due to the greater stability of the selanyl radical ($Se^\bullet$) relative to a thiyl radical ($S^\bullet$), preventing a damaging radical cascade with the C-terminal peptide extension of the enzyme.[34] This concept is illustrated in Figure 2. It is also possible that interaction of the Cys thiol with hydroxyl radical ($^\bullet OH$) could generate the overoxidized cysteine sulfinic or sulfonic acids, as reported by Xu et al. in their studies with cysteine (free amino acid) treated with radiolytically generated $^\bullet OH$.[35] Additionally, Yim and colleagues have shown molecular oxygen to rapidly react with thiyl radicals to form thiyl peroxyl radicals ($RSOO^\bullet$) ($k = 2 \times 10^9$),[36] a species which can undergo a unimolecular transformation to the sulfonyl radical ($RSO_2^\bullet$), a species most likely in transition to the sulfinic acid or sulfonic acid states via radical chain propagation.

To directly test the hypothesis that the use of Sec in TR enables the enzyme to resist inactivation by one-electron oxidation events, we exposed both the Sec- and Cys-TR enzymes to $^\bullet OH$, a one-electron oxidant, generated by the reaction of EDTA-chelated iron with H_2O_2 (Fenton reaction). We first assessed the inactivating effects of "Fe-oxidant", which is generated by Fe-EDTA in the absence of H_2O_2, on the Cys- and Sec-TR enzymes. Chelated iron in the presence of oxygen and absence of H_2O_2 has been demonstrated to form oxidants capable of damaging antioxidant proteins.[30] As shown in the

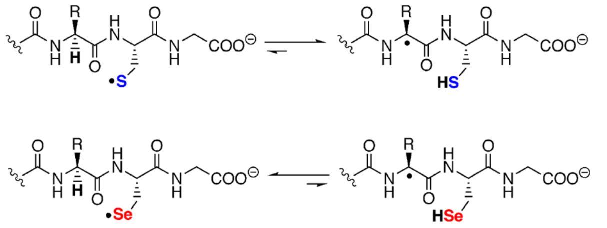


Figure 2. Different stabilities of analogous RS$^{\bullet}$ and RSe$^{\bullet}$ radicals in the C-terminal redox center of TR may lead to protection of the Sec-containing TR from inactivation from a free radical chain reaction.[34] The stabilities of RS$^{\bullet}$ and RSe$^{\bullet}$ radicals are believed to be considerably different, resulting in differing fates of Cys and Sec residues exposed to radical oxidants. The RS$^{\bullet}$ radical (shown in blue) can be deleterious to peptides and proteins due to the favorable abstraction of the Cα-proton (shown in bold). The generated carbon-centered radical can react with oxygen to form peroxyl radicals, initiating peptide degradation. Conversely, the analogous RSe$^{\bullet}$ radical (shown in red) is quite stable, and the proton abstraction reaction is not favored. Figure adapted from ref 34.

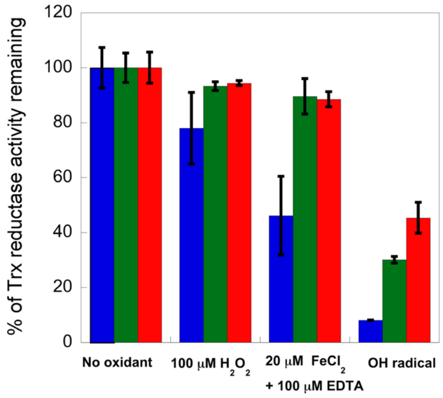


Figure 3. Sec-TR resists inactivation by one-electron oxidants. Prereduced TR enzymes were incubated with either 100 μM H_2O_2 only, 20 μM $FeCl_2$-100 μM EDTA only (Fe-oxidant) or 20 μM $FeCl_2$-100 μM EDTA-100 μM H_2O_2 (Fenton reaction), followed by activity assessment after the addition of 90 μM *E. coli* Trx. The blue bars represent the WT Cys-TR from *D. melanogaster* (DmTR-SCCS), the green bars represent the DmTR-SCUG "Sec-rescue" enzyme, and the red bars represent the WT Sec-TR from mouse mitochondria.

resistance profile in Figure 3, the Sec-TR completely resists inactivation from the Fe-oxidant while the Cys-TR suffers a significant activity loss with this oxidant species (compare red and blue colored bars). When the Sec-rescue enzyme is treated under identical conditions, the enzyme retains significant activity (Figure 3 green bars), indicating the insertion of Sec protects the enzyme against inactivation from the Fe-generated oxidant. The exact species of this Fe-oxidant is unknown. The unique EPR signature displayed by this oxidant is shown in Figure S2 of the Supporting Information and indicates that this oxidant could be a transient radical. Alternative oxidant species may include tetravalent iron [Fe(IV)], ferryl ion (FeO_2^+), or an iron−peroxo (Fe−OOH) complex as described by Sutton and colleagues.[37] Each of these iron oxidants is poorly characterized yet is often proposed in the literature to exert damaging effects on proteins.

In subsequent experiments, we included H_2O_2 in the reaction also containing Fe-EDTA to generate $^{\bullet}$OH and assessed the ability of the Cys- and Sec-TRs to resist inactivation. The amount of remaining Trx-reductase activity of the TR constructs following exposure to $^{\bullet}$OH is reported in Figure 3, along with the activities of the Fe-oxidant, H_2O_2-only, and untreated control samples. The data indicate that the Cys-TR suffers a significant activity loss when exposed to $^{\bullet}$OH. This is demonstrated by the inhibitory effect that is observed upon inclusion of H_2O_2 in the reaction, relative to the reaction containing Fe-EDTA only. This indicates that while the iron-oxidant partially inhibits the Cys-TR, the more potent $^{\bullet}$OH imparts a much more damaging effect. Also of significance is the fact that treatment of the Cys-TR with 100 μM H_2O_2 did not result in significant activity inhibition, in agreement with results from the H_2O_2 resistance studies reported above, where millimolar H_2O_2 concentrations are required to inflict significant inactivation. This result indicates that the loss in activity seen with Fe-EDTA-H_2O_2 reaction is not due to Fe-oxidant alone, nor H_2O_2-inactivation alone, but rather due to the effects of the $^{\bullet}$OH generated from the Fenton reaction. In comparison to the Cys-TR, the Sec-TR displayed significantly more resistance from $^{\bullet}$OH-mediated inactivation (compare red and blue bars in Figure 3). We next carried out the analogous $^{\bullet}$OH resistance experiment with the DmTR Sec-rescue enzyme. Similar to the Sec-TR, the Sec-rescue TR displayed considerable resistance toward inactivation from $^{\bullet}$OH (green bars in Figure 3). While exposure to $^{\bullet}$OH does result in some activity loss, a significant rescue in remaining Trx-reductase activity is observed relative to the Cys-TR (compare green and blue bars in Figure 3). This rescue effect, consistent with results observed with other oxidants, indicates the inactivating effects of $^{\bullet}$OH on the C-terminal redox center of WT Cys-TR can be reversed or significantly reduced by replacement of the active site Cys-nucleophile with Sec.

Resistance against Inactivation from Peroxynitrite. We next investigated the effect of peroxynitrite ($ONOO^-$) exposure on the abilities of the Cys- and Sec-TR enzymes to remain active. Peroxynitrite is an extremely potent oxidant produced in the body by neutrophils and macrophages via the reaction of nitric oxide (NO$^{\bullet}$) with superoxide radical anion. Peroxynitrite is capable of carrying out oxidation, nitration, or nitrosation of biological macromolecules. Peroxynitrite-mediated oxidation can result in DNA strand breaks,[38,39] nitration of free or protein-bound tyrosine residues,[40] and oxidation/nitration of protein-based sulfhydryl groups.[41,42] While $ONOO^-$ formation can serve beneficial antibacterial effects to the immune response, the ability of this oxidant to damage host tissue is well documented, with $ONOO^-$ having been implicated as a major contributor of cardiac injury in ischemic-reperfused hearts.[43] Previously, the selenoenzyme GPx-1 and other selenium-containing GPx-mimetics have been shown to prevent $ONOO^-$ mediated damage to DNA and other proteins.[44] GPx-1 in the presence of glutathione has also been shown to directly reduce $ONOO^-$ *in vitro*.[45] Additionally, Roussyn and colleagues have shown that ebselen, selenocysteine, and selenomethionine better protect DNA from single-strand break formation than sulfur-containing structural analogues.[46] Arteel and co-workers also demonstrated that mammalian Sec-TR could reduce peroxynitrite in a coupled system containing either selenocystine or ebselen.[47] These collective findings led us to predict that both the mammalian Sec-TR and our chimeric Sec-rescue enzyme could better protect *themselves* from inactivation by $ONOO^-$ exposure, relative to the Cys-TR orthologue from *D. melanogaster*. To test this hypothesis, we carried out $ONOO^-$ inactivation resistance experiments with our Cys- and Sec-TR enzymes. The resistance profiles shown in Figure 4 demonstrate both the Sec-TR and Sec-rescue enzyme retain significantly more Trx-reductase activity in comparison to the Cys-TR when each enzyme was exposed to 50–500 μM $ONOO^-$.

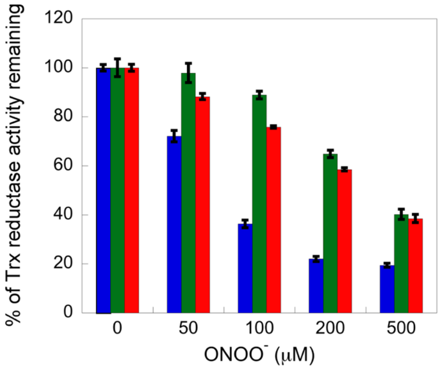


Figure 4. Sec-TR resists inactivation by peroxynitrite. The prereduced TR enzymes were incubated with increasing concentrations of $ONOO^-$ (0–500 μM) then assessed for their ability to reduce 90 μM *E. coli* Trx. The blue bars represent the WT Cys-TR from *D.melanogaster* (DmTR-SCCS), the green bars represent the DmTR-SCUG "Sec-rescue" enzyme, and the red bars represent the WT Sec-TR from mouse mitochondria.

Resistance from Inactivation from Reactive Halogen Species. We next set out to assess the ability of the Cys- and Sec-TRs to resist inactivation by treatment with differing reactive halogen or pseudohalogen species (HOSCN). Hypochlorous (HOCl) and hypobromous (HOBr) acids are powerful oxidants produced in neutrophils and eosinophils, respectively, as part of the host immune defense against invading pathogens.[48] The synthesis of these oxidants is carried out by the myeloperoxidase (MPO)-catalyzed reaction of H_2O_2 with halide ions (Cl^-, Br^-). This enzyme also catalyzes the formation of HOSCN via ^-SCN ions.[49] These hypohalous acids are known to rapidly react with biological macromolecules including protein bound thiols and thioethers, DNA nucleotides, and unsaturated fatty acids.[50] As such, these halogen oxidants have been implicated in a variety of disorders including inflammatory bowel disease and rheumatoid arthritis.[49] The pseudohalogen oxidant HOSCN is less reactive than HOCl and HOBr but has been shown to be considerably more selective for thiols and selenols.[20,49] Figure 5A depicts the resistance profile of the TR enzymes treated with increasing concentrations of HOCl (0–100 μM). The data are consistent with that of other assessed oxidants in demonstrating the remarkably superior resistance from inactivation of the Sec-TRs over the Cys-TR. Specifically, at 100 μM HOCl the Sec-TRs retains ~60% of activity when compared to the untreated control, while the Cys-TR only retains ~20% activity. With HOBr as the oxidant, the differences in resistance are even more

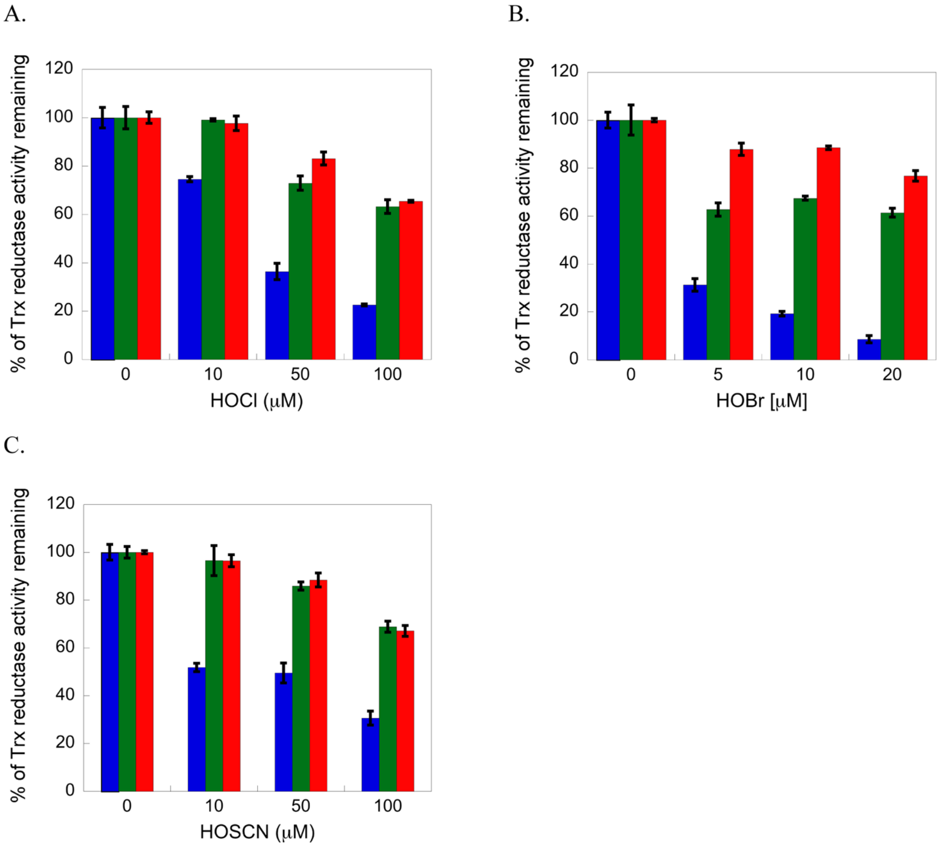


Figure 5. Sec-TR resists inactivation by reactive halogen species and hypothiocyanous acid. The prereduced TR enzymes were incubated with increasing concentrations of oxidant followed by activity assessment with 90 μM *E.coli* Trx. (A) TR enzymes treated with HOCl (0–100 μM). (B) TR enzymes treated with 0–20 μM HOBr. (C) TR enzymes treated with 0–500 μM HOSCN. Blue bars represent the WT Cys-TR from *D. melanogaster* (DmTR-SCCS), green bars represent the DmTR-SCUG "Sec-rescue" enzyme, and red bars represent the WT Sec-TR from mouse mitochondria.

pronounced (Figure 5B). At 5 μM HOBr, the WT Cys-TR retains only ~30% activity while the WT Sec-TR and DmTR-Sec rescue TR retain ~90% and ~65%, respectively. As the concentration of HOBr is increased to 20 μM, the Cys-TR remains only 10% active while both Sec-TRs retain significant activity (~65%). A similar relationship is observed when HOSCN was used as the oxidant species as shown in Figure 5C. The Cys-enzyme only retains ~50% of its activity at 10 μM HOSCN and 30% at 100 μM HOSCN, respectively, while the Sec-TRs are relatively unaffected by 10−50 μM HOSCN and still retain 65−70% of activity at 100 μM HOSCN.

Possible Molecular Mechanisms of Resistance to Inactivation by Oxidation. The mechanism by which TR resists inactivation from HOCl and HOBr is unknown as we failed to find evidence that these oxidants could be used as substrates for the enzyme. However, we, and in collaboration with Dr. Brian Day, report that HOSCN is actually a substrate for the TR enzymes used in this study as summarized by the data compiled in Table 1.[51] The Sec-TR has ~3-fold more HOSCN-reductase activity compared to the Cys-TR and the Sec-rescue TR. Interestingly, both the Cys-TR and the chimeric Sec-rescue TR have similar activities using HOSCN as the substrate. Yet, the Cys-TR becomes inactivated after incubation with HOSCN in the presence of NADPH, while the Sec-rescue enzyme retains activity. We see two possible interpretations of this data. First, during the course of the reaction of the Cys-TR with HOSCN, the active site Cys nucleophile becomes overoxidized to a Cys-SO_2^- residue, and this results in inactivation of the enzyme. The analogous oxidation of Sec to Sec-SeO_2^- in a selenoenzyme is not permanent as discussed in a following paragraph. Second, the addition of a selenium atom to the chimeric Sec-rescue enzyme allows this enzyme to metabolize HOSCN much faster (faster substrate utilization) than the Cys-TR, and this protects some other residue(s) of the enzyme (perhaps the CVNVGC N-terminal redox center) from oxidation and irreversible inactivation. Evidence for the former explanation and against the latter explanation (faster HOSCN clearance) is that both the Cys-TR and the Sec-rescue enzymes utilize HOSCN as a substrate equally well. Though we emphasize that we do not have direct evidence for this hypothesis, such as would be produced by using mass spectrometry.

Table 1. Specific Activities of Sec- and Cys-TR Enzymes with HOSCN as Substrate[a]

enzyme	mol of NADPH min^{-1} mol^{-1} of TR
DmTR-SCCS	540 ± 39
DmTR-SCUG	490 ± 7
mTR3-GCUG	1400 ± 230

[a] The concentration of HOSCN in the assay is 100 μM.

Interestingly, Skaff and colleagues demonstrated the mammalian cytosolic TR1 to be prone to inactivation from treatment with 50−100 μM HOSCN.[20] However, differences in the experimental conditions (use of DTNB as substrate, incubation length) employed in their study as well as differences between TR1 and TR3 may potentially explain this result. The results of Day and co-workers are also in disagreement with the study by Skaff.[51]

Similar to HOSCN, high concentrations of H_2O_2 also inactivate the Cys-TR, but not the Sec-TR or the chimeric Sec-rescue TR as shown by the data in Figure 1. Unlike the case with HOSCN, however, H_2O_2 is a very poor substrate for the Cys-TR as shown by the data compiled in Table 2. Both Sec-containing TRs are able to use H_2O_2 as a substrate, and this could potentially explain why both of these enzymes are resistant to H_2O_2-induced inactivation because the selenium atom imbues these enzymes with the ability to metabolize (turnover) H_2O_2. It should be noted that the Sec-TRs are still poor peroxidases in comparison to GPx-1, which has 10^3−10^4-fold more activity. As such, under conditions of our assay there is a very large excess of H_2O_2 available to damage multiple side chain targets on the enzyme. This fact is supportive of an inactivation mechanism for the Cys-TR that involves formation of Cys-SO_2^- or Cys-SO_3^-. Analogous oxidative end points can also occur with HOCl, HOBr, HOSCN, and $ONOO^-$ oxidants, which would first proceed through intermediary thiol−halous or nitroso−thiol moieties, which would then be readily hydrolyzed to result in the overoxidized sulfinic- or sulfonic-Cys residue.

Table 2. Hydrogen Peroxidase Activity of Semisynthetic DmTR and mTR Enzymes

enzyme	k_{cat} (min^{-1})	K_m (μM)	mol of NADPH min^{-1} mol^{-1} of TR at 50 mM H_2O_2
DmTR-SCCS	N/A[b]	N/A[b]	3.4 ± 0.3
DmTR-SCUG	88.4 ± 3.8	5.5 ± 0.9	82 ± 2
mTR3-GCUG[a]	1753 ± 257	259 ± 46	371 ± 54, 3.4 ± 0.3

[a] Results have been previously reported in ref 22. [b] Not applicable. This enzyme failed to display saturation kinetics.

Evidence that the Sec residue may protect the body of the enzyme by reducing H_2O_2 much more rapidly than if a Cys residue was utilized (oxidant clearance) is presented in Figure S3 of the Supporting Information. We emphasize that even in the case of such rapid oxidant clearance, the Sec residue is still better able to resist permanent inactivation by overoxidation to a Sec-SeO_2^- residue. The two types of protective mechanisms that could be used by Sec discussed above, oxidant clearance or ability of a Sec-SeO_2^- residue to be reduced to the active state, may not be easily separated into distinct types.

We must also consider differences in chemical bonding between sulfur and oxygen and selenium and oxygen that can help explain why a Sec residue resists permanent oxidation. The larger size of selenium and the greater polarizability of its outer electron shell means that these electrons are more available for bonding, hence its greater susceptibility to be oxidized over sulfur.[52] Selenium's greater propensity for bonding with oxygen and reactive oxygen species means that it is a kinetic "target" for oxidants as has been described by Davies.[20] While this is certainly true, it is largely unrecognized in the biochemical literature that selenium and especially *Se*-oxides are much more electrophilic in comparison to sulfur and the corresponding *S*-oxides. In the case of *Se*-oxides, the electrophilic character of the selenium atom is due in part to Se−O bonds that are both longer and weaker than the corresponding S−O bonds. This renders the selenium atom electron deficient and promotes the reduction of the *Se*-oxide back to its fully reduced parent form. Thus Sec-containing enzymes can better resist inactivation by oxidation as further explained in Figure 6.

One last interesting observation from the Sec-rescue enzyme is that the introduction of the selenium atom only gives a rather modest ~2-fold increase in Trx-reductase activity as can be seen

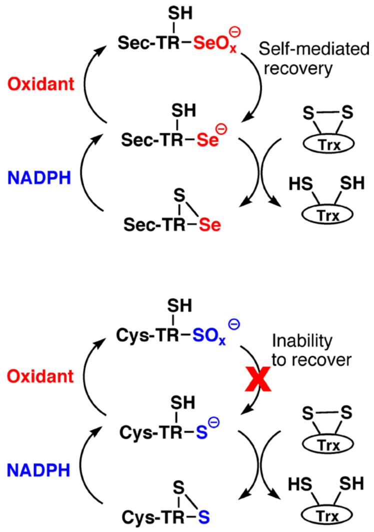


Figure 6. Possible mechanism of resistance to inactivation by oxidation by a Sec-TR. Using NADPH as an electron source, both Cys- and Sec-TRs can reduce the catalytic disulfide bond of Trx. (A) Upon exposure to oxidants, Sec-TR can be oxidized to an inactive form (Sec-TR-SeO$_x$) but can be reduced back to the active selenol/selenolate form via self-mediating reduction by either the adjacent thiol of the C-terminal redox center (shown), or the thiolate of the interchange Cys residue belonging to the C$_{CT}$VNVGC$_{IC}$ N-terminal redox center (C$_{CT}$ is the charge-transfer thiolate and C$_{IC}$ is the interchange Cys). The N-terminal redox center of the enzyme ultimately receives reducing equivalents from NADPH, with FAD/FADH$_2$ acting as the conduit of electrons from NADPH to the N-terminal redox center. The flow of electrons in the enzyme mechanism is NADPH to FAD to N-terminal redox center to C-terminal redox center (GCUG) to substrate (Trx). As long as NADPH is present the N-terminal redox center of the enzyme will remain reduced. A reduced N-terminal redox center should be able to reduce an oxidized Sec residue. (B) The Cys-TR may be inactivated due to irreversible oxidation of the active site Cys nucleophile (Cys-TR-SO$_x$). The Cys-TR has the same molecular architecture as the Sec-enzyme (NADPH binding site/FAD/N-terminal redox center/C-terminal redox center), and the same electron flow from NADPH to the C-terminal redox center (SCCS) but becomes inactivated. This highlights the role of Sec to imbue resistance to inactivation by oxidation in comparison to Cys in the Cys-TR. Higher oxidized forms of Cys, such as Cys-SO$_2^-$ and Cys-SO$_3^-$, are unable to be reduced back to the active thiol/thiolate form. The inability of the Cys-TRs to recover from oxidative insult highlights the important biological role served by Se in Sec-TRs.

by the data compiled in Table 3. This is similar to a ∼4-fold increase in activity reported for citrus phospholipid glutathione peroxidase when the active site Cys was replaced with Sec.[54] While this is a small sample size, it does not support the notion that the use of Sec in an enzyme gives it a very large catalytic advantage that justifies the usage of the bioenergetically costly Sec-insertion system. As shown by the data reported here, the switch from sulfur to selenium does reveal a very large difference in the ability of the respective enzymes to resist inactivation by oxidation that might justify the use of Sec in an enzyme.

Further Evidence That Selenium Confers Resistance to Inactivation by Oxidation from the Literature. Parkin and colleagues showed that the Sec-containing [NiFeSe]-hydrogenases from *Desulfomicrobium baculatum* display greater oxygen tolerance and more rapid reactivation than certain Cys homologues.[55] It is also interesting to note that in a recently solved X-ray crystal structure of the [NiFeSe] hydrogenase from *Desulfovibrio vulgaris* in the "as-isolated" oxidized state the active site contained a coordinating Cys residue as a sulfinate while the neighboring Sec residue lacked such an overoxidation.[56] While the structural significance of this overoxidized Cys residue is unknown, the fact that the Cys residue, but not the nearby Sec residue, was overoxidized may provide support for the superior ability of Sec to recover from oxidative insult.

Table 3. Thioredoxin Reductase Activity of Semisynthetic and Recombinant TR Enzymes

enzyme	k_{cat} (min^{-1})	K_m (μM)
DmTR-SCCS[a]	300 ± 7	173 ± 8
DmTR-SCUG	537 ± 61	94 ± 23
mTR-GCUG[b]	2220 ± 78	67.6 ± 6
DmTR-SCUG[c]	∼900	∼7

[a]Results for the WT recombinant DmTR enzyme have previously been reported in ref 22. [b]Results for the WT semisynthetic mTR enzyme have previously been reported in ref 23. [c]Results with the recombinant DmTR-SCUG have been reported in ref 53. This enzyme was assayed against WT DmTrx-2 instead of *E. coli* Trx as reported in our study.

As briefly discussed above, the use of Sec may not be confined to a strictly catalytic role and may serve in the role of a protective antioxidant. An example of such a protective role was recently reported in the Seleno CYP119, a Sec mutant of the thermophilic cytochrome P450 enzyme from *Sulfolobus acidocaldarius*.[57] The authors aimed to explore the effect of Sec for Cys substitution on electron donation with respect to the catalytic cycle. Interestingly, in studies in which both the Se CYP119 (Sec mutant) and WT Cys CYP119 enzymes were treated with 10 equiv of *m*-chloroperbenzoic acid, a peroxycarboxylic acid oxidant, the Se CYP119 enzyme suffered a 40% loss in total heme content while the WT Cys enzyme lost greater than 90% of its heme content. This observation suggested the Se CYP119 enzyme was much more resistant to oxidant induced heme loss than was the WT Cys enzyme. Coincidentally, Yamashita and co-workers have reported that selenoneine, an organoselenium small molecule found in high amounts in tuna, binds the heme moiety of hemoglobin and myoglobin, effectively protecting these proteins from autooxidation by iron ions.[58] Last, the Sec residue of the artificial selenoenzyme selenosubtilisin has been shown to be oxidized to the Sec-SeO$_2^-$ form. The activity of this inactive form of the enzyme can be restored by the addition of thiol reducing agents.[59]

■ CONCLUSION

Our results clearly indicate that Sec-TRs are able to resist significant inactivation under oxidative conditions in which an orthologous Cys-TR is largely inactivated under identical conditions of oxidative stress. This relationship was true for each of the reactive oxygen species, reactive nitrogen species, and reactive halogen oxidants investigated in this report. As we have discussed in previous reports, we continue to believe that the use of Sec in an enzyme is not due to a catalytic advantage that it enjoys relative to the use of Cys, but rather due to a chemicobiological advantage it provides with respect to resistance to irreversible inactivation by oxidation.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1: plots of absorbance vs time for the hydrogen peroxidase assays as an example of all such dat; Figures S2: EPR spectra of one-electron oxidant species; Figure S3: DTNB time-course reactions for the enzymes used in this study after exposure to 50 mM H_2O_2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

These studies were supported by National Institutes of Health Grant GM094172 to R.J.H.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

A_{340}, absorbance at 340 nm; A_{412}, absorbance at 412 nm; Cys, cysteine; Cys-SO_2^-, sulfinic acid form of Cys; Cys-SO_3^-, sulfonic acid form of Cys; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DmTR, *Drosophila melanogaster* thioredoxin reductase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; $FeCl_2$, ferrous chloride; Gly, glycine; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; HOBr, hypobromous acid; HOSCN, hypothiocyanous acid; KSCN, potassium thiocyanate; LPO, lactoperoxidase; M_r, molecular ratio; mTR, mouse thioredoxin reductase; NADPH, *β*-nicotinamide adenine dinucleotide phosphate-reduced; $^\bullet$OH, hydroxyl radical; $ONOO^-$, peroxynitrite; $S^\bullet$, thiyl radical; $Se^\bullet$, selanyl radical; Sec, selenocysteine; Sec-SeOH, selenenic acid form of Sec; Sec-SeO_2^-, seleninic acid form of Sec; Sec-SeO_3^-, selenonic acid form of Sec; Ser, serine; TNB, thionitrobenzoic acid; Trx, thioredoxin; TR, thioredoxin reductase; U, the one letter code for Sec; WT, wild type.

■ ADDITIONAL NOTE

[a]The mouse mitochondrial TR is abbreviated as mTR3-GCUG. The abbreviation mTR refers to mouse thioredoxin reductase, and the letters GCUG refer to the sequence of the last four amino acids in the C-terminal redox center, glycine-cysteine-selenocysteine-glycine (GC<u>U</u>G). The number 3 denotes the cellular compartment (mitochondria). In this nomenclature system, mTR1 is the cytosolic enzyme and mTR2 is the testes specific TR. The cytosolic Cys-orthologue from *D. melanogaster* is abbreviated DmTR-SC<u>C</u>S, with the letters SCCS referring to the C-terminal tetrapeptide motif serine-cysteine-cysteine-serine. The chimeric DmTR "Sec-rescue" enzyme is abbreviated as DmTR-SC<u>U</u>G because the active site **<u>C</u>** has been replaced with Sec (**<u>U</u>**). Truncated enzymes missing the final 8 amino acids are abbreviated as mTRΔ8 and DmTRΔ8.

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