Chapter 6
Chemical Basis for the Use of Selenocysteine

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Abstract Since the discovery of selenocysteine as the 21st amino acid in the genetic code, two streams of thought have dominated the question of why selenium is used to replace sulfur in enzyme active sites in the form of selenocysteine. These ideas are that selenocysteine is (i) a "relic of the anaerobic world" and (ii) "catalytically superior" to the use of sulfur as cysteine. This latter idea is due to the experimental finding that the replacement of selenocysteine with cysteine in enzyme active sites results in a large drop in catalytic activity, and has been interpreted to mean that selenocysteine is essential for catalyzing the formation of product from substrate. We and others have previously proposed that selenocysteine is not catalytically essential since cysteine homologs of selenocysteine enzymes exist and catalyze their enzymatic reactions with comparable efficiency. Here, and elsewhere, we discuss the idea that the use of selenocysteine confers an enzyme with the ability to resist irreversible inactivation by oxidation.

6.1 Introduction

Selenocysteine (Sec, U) is distinct from the other 20 common proteinogenic amino acids due to the complexity of its insertion into the polypeptide chain, which involves recoding of a stop codon as a sense codon, the use of a *cis*-acting factor in the mRNA, and multiple protein accessory factors [1]. The elaborate nature of the recoding process likely indicates that Se has a unique chemical function that the S-atom of cysteine (Cys) cannot fulfill.

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What is the special chemical function of Se that explains its use in enzymes? A popular idea is that Sec is "catalytically superior" to the use of Cys in enzymes and is necessary for the conversion of substrate to product. This idea likely originated from the fact that mutation of the active-site Sec residue in enzymes to Cys results in large drop in the catalytic rate constant (k_{cat}) [2, 3]. Another early experiment that lent support for this idea was the substitution of Sec into a naturally occurring Cys-enzyme. In the case of a Cys-containing phospholipid hydroperoxidase, this Sec for Cys substitution resulted in an enzyme with higher catalytic activity than the wild type enzyme [4]. However, it was later shown by Stadtman that the Sec-containing selenophosphate synthetase from *H. influenzae* did not have higher catalytic activity than the Cys-containing ortholog from *E. coli* [5]. This result led Stadtman to suggest "…a role of selenocysteine in *H. influenzae* that is not catalytic." This latter idea has not been widely championed in the field.

A specific catalytic role for Se in enzymes has been difficult to discern because most of the physico-chemical properties of Se and S are quite similar as has been noted in a recent review [6]. Many researchers have focused on the differences in nucleophilicity and acidity between Se and S as rationales for the use of Sec in enzymes [7]. As we have pointed out in a recent review article, when Se and S have equal ionization states, the ratio of Se nucleophilicity to S nucleophilicity is modest, in the range of 5–10 [8]. There are also multiple examples of Cys residues in enzymes with pK_a values lower than 5 [8]. Despite these high similarities between Se and S, my research group (and others) has tried to determine a specific catalytic role for Se in the enzyme thioredoxin reductase (TR). We initially focused on what we have termed as a "niche rationale" for the use of Se in TR by explaining its role in the catalytic cycle as a superior leaving group (due to a lower pK_a) and stabilizing a certain conformer of the enzyme due to longer C–Se and Se–S bond lengths [9]. However, as more experimental evidence accumulated, we began to question this model for the role of Se in TR (and other enzymes) and started to focus on a role for Se that is not catalytically essential as originally suggested by Stadtman as well as others [10].

While there is no question that Se plays an important mechano-chemical function in the enzymes where it occurs (we refer to the chemical property of Se that supports conversion of substrate to product as its mechano-enzymatic function), we, and others, argue that S can compensate for the absence of Se in enzyme active sites due to chemical tuning of the active-site microenvironment. We hypothesized that the mechano-enzymatic function of Se should be related to its non-catalytic, biological function in enzymes [8]. Review of our mechanistic experiments led us to posit that the electrophilicity of Se was the mechano-enzymatic function that allowed TR to convert substrate to product. At the same time, electrophilicity of the Se atom also allows it (and the enzyme) to resist irreversible inactivation by oxidation as discussed below.

One possible way in which a selenoenzyme can resist irreversible inactivation by oxidation is shown in Fig. 6.1. Both Cys- and Sec-enzymes require a reduced thiol or selenol in their respective active-sites to be in the active, functional state. Upon exposure to oxidant (such as H_2O_2), both enzymes can be oxidized to inactive forms as either the sulfenic (Enz-SOH) or selenenic acid (Enz-SeOH) oxidation states. Both the Enz-SOH and Enz-SeOH forms can be reduced back to the active state

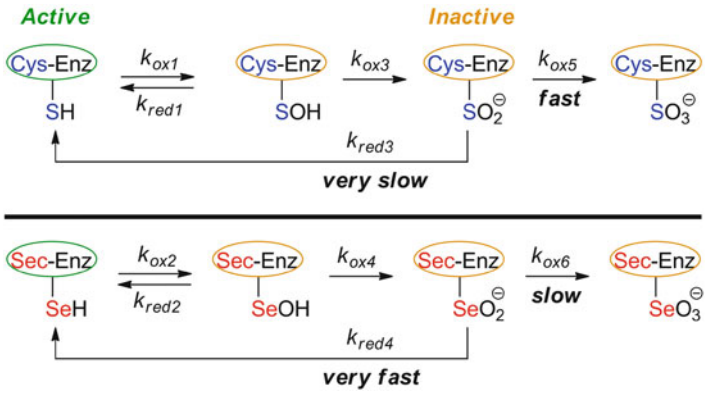


Fig. 6.1 Cys- and Sec-enzymes require a reduced thiol or selenol, respectively, to be in the active, functional state. Each enzyme can be oxidized by H_2O_2 to inactive Enz-SOH and Enz-SeOH forms, respectively, with $k_{ox2} > k_{ox1}$. Reduction by thiol restores both inactive forms back to the active state with $k_{red2} > k_{red1}$. Addition of a second equivalent of H_2O_2 to Enz-SOH and Enz-SeOH oxidation states leads to formation of Enz-SO_2^- and Enz-SeO_2^-, respectively. Presumably $k_{ox4} > k_{ox3}$, though this has not been experimentally determined. However, $k_{red4} >> k_{red3}$, with the sulfinic acid being extremely resistant to reduction. The Enz-SeO_2^- form resists further oxidation to Enz-SeO_3^-, while Enz-SO_2^- is oxidized to Enz-SO_3^- relatively easily. In this case, $k_{ox6} << k_{ox5}$

by the addition of exogenous thiol. A key chemical difference between Cys- and Sec-enzymes is revealed when the two enzymes are oxidized to the sulfinic acid (Enz-SO_2^-) and seleninic acid (Enz-SeO_2^-) forms. The sulfinic acid form of a Cys-enzyme cannot be chemically reduced back to Enz-SH by the addition of thiols such as glutathione because the S-atom of Enz-SO_2^- is not very electrophilic. In contrast, the Enz-SeO_2^- form of a selenoenzyme can be readily reduced back to Enz-SeH, as has been shown by the work of Hilvert and coworkers through their study of seleno-subtilisin [11]. The reason for the much faster reduction of $RSeO_2^-$ compared to RSO_2^- is because Se is much more electrophilic than S [12, 13], and it is this superior ability of Se to accept electrons relative to S that can explain both its mechano-enzymatic function and its chemico-biological (non-catalytic) function in enzymes.

A second chemical difference between Se and S that can help a selenoenzyme resist irreversible inactivation by oxidation is the fact "that while S(VI) is a stable oxidation state for sulfur relative to S(IV) exactly the reverse is true for Se(VI) vs. Se(IV)" [14]. As shown in Fig. 6.1, this means that it is *more difficult* to further oxidize the Enz-SeO_2^- form of a Sec-enzyme to the selenonic acid form (Enz-SeO_3^-) than is the same oxidation of the Enz-SO_2^- form of a Cys-enzyme to the sulfonic acid form (Enz-SO_3^-). The reason for the slower oxidation of Se(IV) to Se(VI) is also related to the electropositive character of Se. The lone pair of electrons on $RSeO_2^-$ is not readily available for bonding, because they are strongly attracted to the positive Se nucleus. In contrast, the lone pair of electrons on RSO_2^- is readily available for nucleophilic attack onto electrophiles as shown by the reaction of *p*-toluensulfinic acid with benzeneseleninic acid [14]. In this reaction, the Se atom acts as the electron acceptor and S acts as the electron donor, the opposite of what is commonly thought about the nucleophilic character of Se.

6.2 Chemical Models to Study the Oxidation States of S and Se

We wished to quantify the rates of oxidation and reduction of Enz-SO_2^- and Enz-SeO_2^- in enzyme active sites. In order to simplify the problem, we chose small molecule S- and Se-model compounds to determine these rates. These compounds are benzenesulfinic acid ($PhSO_2^-$) and benzeneseleninic acid ($PhSeO_2^-$), respectively. A simple qualitative difference between the two compounds was immediately obvious to us upon addition of excess β-mercaptoethanol (βME) to both compounds. In the case of $PhSeO_2^-$, an immediate yellow precipitate formed upon the addition of βME (Fig. 6.2). We determined by mass spectrometry that this yellow precipitate was PhSe–SePh. In contrast, there was no evidence of reaction upon addition of βME to $PhSO_2^-$.

These reactions were more carefully followed using ^{1}H-NMR. The reduction of both the sulfinic and seleninic acids were carried out under an Ar atmosphere in K_2HPO_4/KH_2PO_4 buffered D_2O (Ar sparged) that was 50 mM in substrate. After obtaining an initial ^{1}H-NMR spectrum, βME was added (1.0–5.0 Eq). The observation of precipitate in the case of the seleninic acid substrate was also apparent in the ^{1}H-NMR spectrum due to the lack of aromatic signals (Fig. 6.2c). In order to slow the rate of reduction, the reaction was carried out in deuterated methanol at −65°C (data not shown). However, even at −65°C the reduction of the seleninic acid to the selenosulfide was too fast to be observed by ^{1}H-NMR. In contrast, the reduction of the sulfinic acid with βME was so slow that no reaction was observed after 2 weeks at room temperature (Fig. 6.2f). In order to increase the rate of the reduction of $PhSO_2^-$, the reaction was carried out in deuterated methanol at 85°C (data not shown). Even at this elevated temperature, the reduction of sulfinic acid was not observed even after 2 weeks.

The *oxidation* of both the sulfinic and seleninic acids to their respective sulfonic and selenonic forms were carried out under an Ar atmosphere in K_2HPO_4/KH_2PO_4 buffered D_2O (Ar sparged) that was 50 mM in substrate. After obtaining an initial ^{1}H-NMR spectrum, H_2O_2 was added (1.0 Eq for $PhSO_2^-$, while 10.0 Eq for $PhSeO_2^-$). The oxidation was monitored by ^{1}H-NMR at specific time points depending on the rate of substrate oxidation (minutes for $PhSO_2^-$ substrate, while days for $PhSeO_2^-$ substrate). In both cases the rate of substrate oxidation was determined as $\text{rate} = k[\text{substrate}]^1[H_2O_2]^1$. For the $PhSO_2^-$ substrate, the second order rate constant was determined by a plot of $[PhSO_2^-]^{-1}$ vs. time (s). Since the concentration of both $PhSO_2^-$ substrate and H_2O_2 are the same, the slope of the line obtained is the second order rate constant (k). For the $PhSeO_2^-$ substrate, the second order rate constant was determined using pseudo-first order kinetics due to the excess H_2O_2 required to promote oxidation. A plot of $\ln[PhSeO_2^-]$ vs. time (s) provided the pseudo-first order constant (k'). Since $k' = k[H_2O_2]_0$, the second order rate constant (k) could then be calculated. The ^{1}H-NMR spectra of the oxidation of $PhSeO_2^-$ and $PhSO_2^-$ by H_2O_2 monitored over time are shown in Fig. 6.3 (top and bottom, respectively).

As can be seen in the time courses, the oxidation of $PhSeO_2^-$ is very slow (even with 10 Eq of H_2O_2) and the oxidation of $PhSO_2^-$ is relatively fast, especially at acidic pH. While the reduction of each compound proved either to be too fast

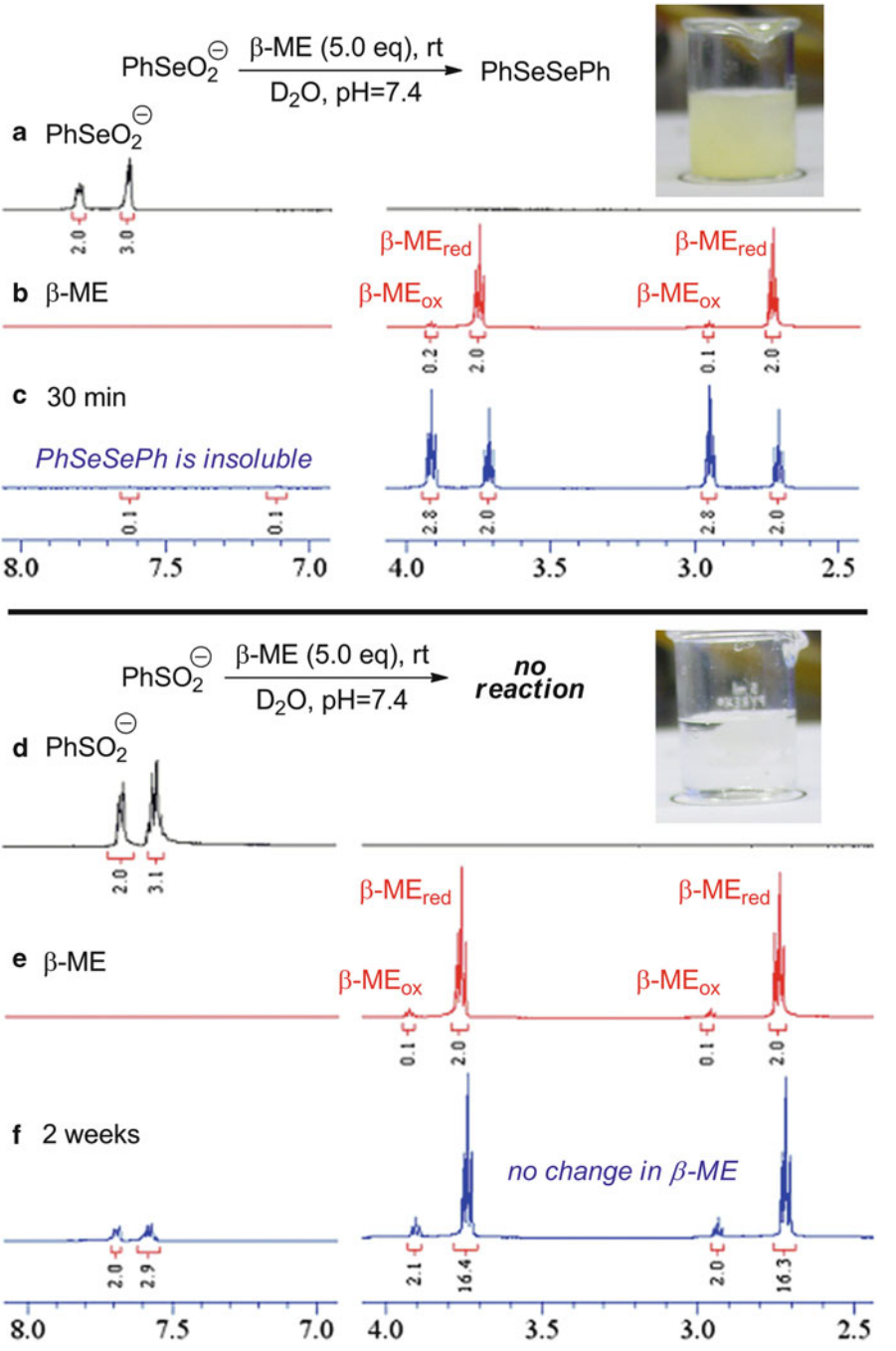


Fig. 6.2 The reduction of $PhSeO_2^-$ (**a–c**) and $PhSO_2^-$ (**d–f**) by βME monitored by 500 MHz ^{1}H-NMR. Upon addition of βME, PhSe-SePh is produced directly as a *yellow* precipitate (beaker in *upper right corner* of figure). This is not only visibly apparent in the reaction flask, but also in the ^{1}H-NMR spectra (**c**) due to the lack of aromatic signals. The reduction of $PhSO_2^-$ differs significantly as shown by the absence of reaction after 2 weeks (**f**). Compare the two reductions qualitatively by examining the reaction flasks

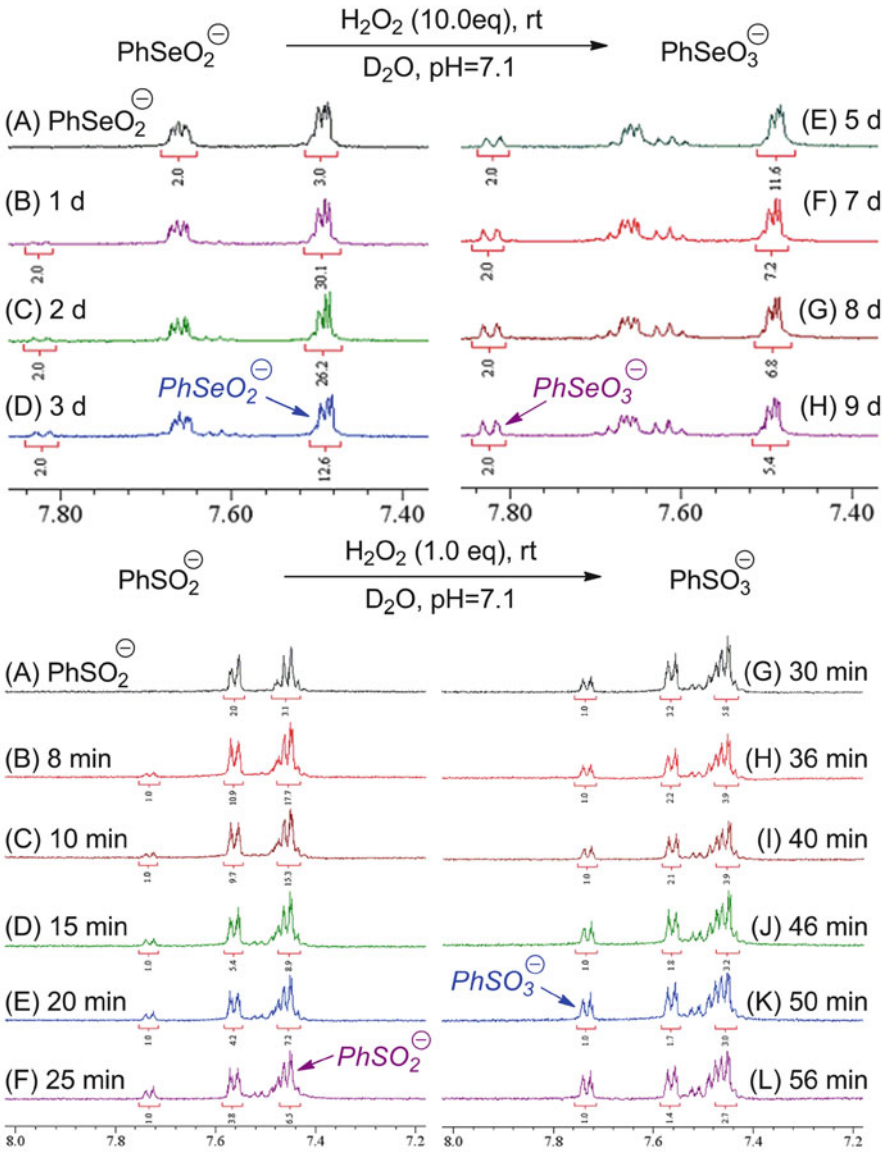
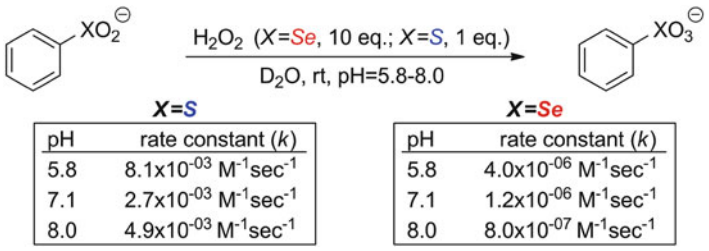


Fig. 6.3 The oxidation of $PhSeO_2^-$ (*top*) and $PhSO_2^-$ (*bottom*) by H_2O_2, monitored by a 500 MHz ^{1}H-NMR. For the oxidation of $PhSeO_2^-$, it takes a 10 Eq excess of H_2O_2 over 9 days to reach an approximately 50:50 mixture of $PhSeO_2^-$ to $PhSeO_3^-$. In contrast, the oxidation of the sulfur analogue requires only 1 Eq of H_2O_2 and 1 h reaction time to reach an approximately 50:50 mixture of $PhSO_2^-$ to $PhSO_3^-$



Scheme 6.1 Summary of experimentally determined rate constants at various pH values for the oxidation of $PhSeO_2^-$ and $PhSO_2^-$ to $PhSeO_3^-$ and $PhSO_3^-$, respectively. The oxidation of the S-compound was much faster than the Se-compound at all pH values, with the difference at acidic pH being the largest

($PhSeO_2^-$) or too slow ($PhSO_2^-$) to determine a rate constant using ^{1}H-NMR, the oxidations were on a time scale that allowed for determining oxidation rate constants and this data is summarized in Scheme 6.1. The data shows that the oxidation of $PhSO_2^-$ is ~2,200-fold faster than $PhSeO_2^-$ at pH 7.1 and ~2,000-fold faster at pH 5.8. We note that while we were not able to determine a rate constant for the reduction of $PhSeO_2^-$, Hilvert and coworkers were able to measure an observed rate constant for the reduction of a model seleninic acid compound ($RSeO_2^-$) using stopped-flow techniques. This rate constant was determined to be 3.3×10^6 M^{-1} min^{-1} [11]. Moreover, they showed that the pH optimum of this reduction was between 4 and 5. This increased rate of reduction at acidic pH parallels our own experiments using methaneseleninic acid as a substrate for a truncated TR missing the C-terminal Sec residue [15]. Thus, the data clearly shows very large differences in the chemistries of the oxides of Se and S; seleninic acid is reduced exceptionally fast by a thiol (especially at acidic pH) and sulfinic acid is reduced exceedingly slow. Based on our data, we estimate that the ratio of the rates of reduction of $PhSeO_2^-$ to $PhSO_2^-$ is $\geq 10^6$ (k_{red4}/k_{red3} in Fig. 6.1). Conversely, the oxidation of seleninic acid is relatively slow compared to the same oxidation of sulfinic acid (especially at acidic pH), and this is the basis for our assertions outlined in Fig. 6.1.

6.3 Sec-Containing Thioredoxin Reductase Resists Inactivation by Oxidation

We recently set out to test our hypothesis that Sec-enzymes resist irreversible inactivation by oxidation [15]. The results showed that mouse mitochondrial Sec-TR resisted inactivation from exposure to 50 mM H_2O_2 as shown in Fig. 6.4a. Our hypothesis predicts that a Sec-enzyme will be more resistant to inactivation by oxidation than a Cys-enzyme as outlined in Fig. 6.1. To test this prediction we tested

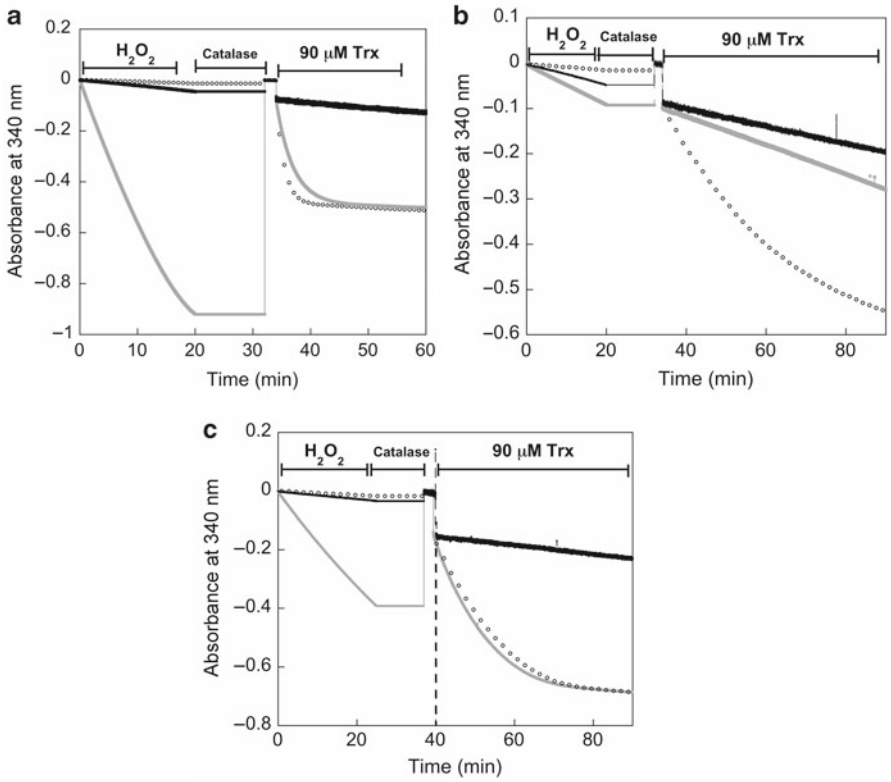


Fig. 6.4 Resistance of SecTR to inactivation by H_2O_2. (**a**) Exposure of mammalian SecTR-GCUG to 50 mM H_2O_2. For this experiment, the enzyme is incubated with (*grey line*) and without (*open circles*) 50 mM H_2O_2. The *black line* is the nonenzymatic control (no enzyme). The reaction progress is monitored by measuring the consumption of NADPH at 340 nm. After 20 min of incubation with 50 mM H_2O_2, catalase is added to consume the remaining H_2O_2 (12 min), after which 90 μM *E. coli* Trx is added to the reaction assay to assess if the Trx-reductase activity of the enzyme is affected [15]. The reaction progress curves of both peroxide treated and untreated are essentially the same. The same experiment (**b**) is repeated for wild type DmTR-SCCS (Cys-DmTR). The results show that the Trx-reductase activity of Cys-DmTR is greatly affected by exposure to 50 mM H_2O_2 (compare *open circles* to *grey line*). (**c**) When Sec is substituted for Cys in the DmTR-SCUG mutant (Sec "rescue"-TR), the enzyme becomes resistant to inactivation by peroxide (compare *open circles* to *grey line*)

the ability of a Cys-ortholog of mammalian TR to resist inactivation by exposure to H_2O_2. This ortholog is the Cys-containing TR from *D. melanogaster* (DmTR), which contains a C-terminal SC_1C_2S redox motif instead of the GC_1U_2G redox motif found in mammalian TR. The results of this experiment are shown in Fig. 6.4b [16], they show that the Cys-TR is significantly inactivated by exposure to 50 mM H_2O_2. Our hypothesis implies that replacement of the Cys residue in DmTR with a Sec residue should reverse this inactivation. We then constructed a mutant of DmTR in

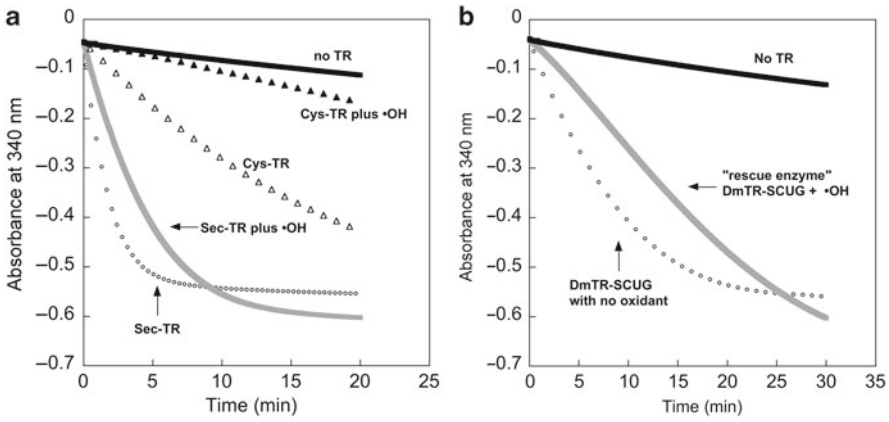


Fig. 6.5 Resistance of SecTR to inactivation by hydroxyl radical. (**a**) Exposure of mammalian SecTR-GCUG to •OH generated by Fe•EDTA/H_2O_2 in situ. Here, the enzyme is incubated with (*grey line*) and without (*open circles*) •OH followed by a quenching step. Trx is then added to the reaction mixture and activity is measured by loss of absorbance at 340 nm. The *black line* is the nonenzymatic control (no enzyme). While mammalian Sec-TR resists inactivation by •OH the Cys-ortholog (DmTR-SCCS) is largely inactivated as can be seen by comparing activity of the enzyme without •OH (*open triangles*) to the enzyme activity after •OH treatment (*closed triangles*). The presence of Se in the Sec "rescue"-TR (the DmTR-SCUG mutant) confers resistance to oxidation by •OH as shown by the plot depicted in (**b**). Compare DmTR-SCUG with •OH treatment (*grey line*) with DmTR-SCCS without •OH treatment (*open circles*)

which we replaced the active-site Cys_2 residue (the Cys residue in the second position of the dyad) with a Sec residue using protein semisynthesis [17]. Thus we have a pair of enzymes termed Cys-DmTR (with C-terminal sequence of SC_1C_2S) and Sec-DmTR (or also called Sec "rescue"-TR with C-terminal sequence of SC_1U_2G) that differ in sequence by only a single atom from ~6,000 atoms in each subunit. We then tested the ability of Sec-DmTR to resist inactivation by 50 mM H_2O_2. The results are shown in Fig. 6.4c. As can be seen from the data, the substitution of Sec for Cys confers the mutant enzyme with the ability to resist inactivation by oxidation from H_2O_2 as our hypothesis predicts.

Concomitant with the publication of our hypothesis that Sec-enzymes would resist irreversible inactivation by oxidation, Koppenol and coworkers also predicted that Sec-enzymes would resist inactivation by oxidation, with a specific prediction that Sec-enzymes would resist one-electron oxidations due to the high stability of a selanyl radical (RSe•) relative to a thiyl radical (RS•) ([18] and see also [19]). Indeed, they showed that the selanyl radical was more stable than the thiyl radical by a factor of 10^{10}. We tested this specific hypothesis with our Cys-DmTR and Sec-DmTR system mentioned above using the hydroxyl radical (•OH) as the one-electron oxidant. The hydroxyl radical was generated using Fenton chemistry with Fe•EDTA/H_2O_2 as the source of the radical. As shown in Fig. 6.5a, Cys-DmTR was largely

inactivated by •OH while the Sec-mitochondrial TR greatly resisted this inactivation. Similar to our results with H_2O_2, the Sec-"rescue" enzyme (DmTR-SCUG) gained the ability to resist inactivation by •OH as shown in Fig. 6.5b. The experimental results exactly match the prediction by Koppenol and coworkers.

The exact sequence of chemical events is not known that allows the Sec-TR to survive a one-electron oxidation reaction. Presumably, Se can donate a hydrogen atom (or an electron from the selenolate) to •OH allowing for the formation of H_2O. The resulting Enz-Se• radical could then react with a second molecule of •OH, forming Enz-SeOH. This selenenic acid form of the enzyme can then either be reduced back to Enz-SeH or further oxidized to Enz-SeO_2^-. In the case of TR, if the seleninic acid form is produced, it will quickly be reduced back to the selenol [15] and this seleninic form will strongly resist further oxidation as our experiments have shown. This property of Se allows the enzyme to survive catastrophic degradation of the peptide backbone [18]. In contrast, if S replaced Se, the formed thiyl radical would react by abstracting a H• radical from the peptide backbone, initiating a radical catalyzed degradation cascade of the peptide backbone with concomitant loss in enzyme activity [18]. One-electron oxidations of S can also result in the formation of RSO_2^- [20], or Enz-SO_2^-, which would irreversibly inactivate the enzyme.

6.4 Conclusions

As discussed here and elsewhere, we have hypothesized that the use of Sec in enzymes is due to factors other than for supporting efficient catalysis, e.g., that Sec is catalytically necessary to convert substrate to product. Previously [15], and as presented here, the data demonstrates that the presence of a Sec-residue in TR imbues the enzyme with the ability to resist irreversible inactivation by oxidation. This idea was originally proposed by Rocher and coworkers, though expressed in a different way [21]. Rocher proposed that the use of Se in glutathione peroxidase was to prevent "significant self-inactivation" due to reaction with hydroperoxides. Tolerance toward oxygen induced inactivation has also been proposed as a rationale for the use of Se in place of S in the NiFeSe cluster of a bacterial hydrogenase [22]. Resistance to irreversible oxidation comes in two forms: (i) the ability of Se-oxides to be recycled back to the parent selenol as shown in Fig. 6.1, and (ii) the ability of Se to resist inactivation by one-electron oxidation by one-electron oxidants. The former property would be advantageous in the "aerobic world," while the latter property would be advantageous in enzymes that might be exposed to one-electron oxidation events. One-electron oxidation events do not depend on the presence of oxygen and this could possibly explain the initial appearance of Sec in the "anaerobic world." Finally, we note that our hypothesis is not yet definitively accepted in the field and that multiple rationales may exist for the use of selenium in enzymes.

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