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Using chemical approaches to study selenoproteins - focus on thioredoxin reductases

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Abstract

The study of selenocysteine-containing proteins is difficult due to the problems associated with the heterologous production of these proteins. These problems are due to the intricate recoding mechanism used by cells to translate the UGA codon as a sense codon for selenocysteine. The process is further complicated by the fact that eukaryotes and prokaryotes have different UGA recoding machineries.

This review focuses on chemical approaches to produce selenoproteins and study the mechanism of selenoenzymes. The use of intein-mediated peptide ligation is discussed with respect to the production of the mammalian selenoenzymes thioredoxin reductase and selenoprotein R, also known as methionine sulfoxide reductase B1. New methods for removing protecting groups from selenocysteine post-synthesis and methods for selenosulfide/diselenide formation are also reviewed.

Chemical approaches have also been used to study the enzymatic mechanism of thioredoxin reductase. The approach divides the enzyme into two modules, a large protein module lacking selenocysteine and a small, synthetic selenocysteine-containing peptide. Study of this semisynthetic enzyme has revealed three distinct enzymatic pathways that depend on the properties of the substrate. The enzyme utilizes a macromolecular mechanism for protein substrates, a second mechanism for small molecule substrates and a third pathway for selenium-containing substrates such as selenocysteine.

Keywords

selenocysteine; peptide ligation; semisynthesis; seleno effect; thioredoxin reductase; vicinal disulfide

INTRODUCTION

Proteins containing selenocysteine (Sec) – selenoproteins, are among the most difficult type of proteins to produce using recombinant DNA technology due to the fact that the sense codon for selenocysteine (Sec) is also the opal (UGA) stop codon [1]. The UGA stop codon is read as a Sec codon when a *cis* acting factor, a stem-loop structure designated as a selenocysteine insertion sequence (SECIS) element is present in the same piece of mRNA [2,3]. In addition to the SECIS element, a number of *trans* acting factors are also required. In *E. coli*, these

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include: Sec synthase (SelA), which synthesizes Sec on a specialized serine tRNA molecule (SelC), a Sec-specific elongation factor (SelB), and selenophosphate synthetase (SelD) that produces monoselenophosphate from a selenide donor [4]. Böck demonstrated that the “barriers” to heterologous production of selenoproteins in *E. coli* were due to the fact that SelB poorly recognized the SECIS element from the heterologous piece of mRNA [5]. In addition, a heterologously expressed SelB from *D. baculatum* that was co-expressed with the mRNA of *D. baculatum hydV* in *E. coli* was insufficient to insert Sec into the hydrogenase large subunit. Apparently the heterologous SelB protein does not interact correctly with the *E. coli* ribosome. The heterologous expression of eukaryotic proteins in bacteria is further complicated by the difference in the structures of eukaryotic and prokaryotic SECIS elements as well as the placement of the SECIS element relative to the UGA codon in these two domains of life (see Figures 1,2, and 3 of citation [6]).

Despite these large problems, biotechnological solutions have been developed, first led by Böck, and later by Arnér and coworkers. Böck first demonstrated that a heterologous selenoprotein could be produced in *E. coli* by constructing a chimeric mRNA. This chimeric mRNA consisted of the mRNA for formate dehydrogenase from *M. formicicum*, containing a UGA codon in place of the normal UGC (Cys) codon, with a correctly placed *E. coli fdhF* SECIS element [7]. This same strategy was used for the heterologous production of the eukaryotic citrus phospholipid hydroperoxide glutathione peroxidase in *E. coli* for the purpose of a Sec for Cys substitution [8]. This was accomplished by overexpressing the SelC gene product on the same plasmid for the mutant peroxidase as well as an engineered version of the *E. coli fdhF* SECIS element. While Sec insertion was successful, several mutations of the protein also resulted due to conservation of the in-frame SECIS sequence. The engineered Sec-peroxidase did have higher activity than the wild type (WT) enzyme due to the presence of the more chemically reactive selenol functional group.

Arnér and coworkers developed the first biotechnologically useful expression system of a mammalian selenoprotein by demonstrating the high level expression of the mammalian selenoenzyme thioredoxin reductase (TrxR) in *E. coli* cells [9]. The Sec residue of TrxR is the penultimate amino acid found in a conserved Gly-Cys-Sec-Gly redox tetrapeptide motif [10]. The position of the Sec residue in TrxR made it an attractive target for recombinant expression of a mammalian selenoenzyme. For this accomplishment, Arnér's group also used an engineered version of the *E. coli fdhF* SECIS element that overlapped the 3' end of the TrxR gene. Production of the recombinant TrxR was increased by overexpressing the SelA, SelB, and SelC gene products and supplementing the growth medium with sodium selenite. The production of recombinant TrxR was later enhanced by suppressing the production of release factor 2 (RF2) and optimizing growth culture conditions [11]. This optimized approach yielded 40 mg of rat TrxR per liter of bacterial culture with 50% of the protein sample containing Sec. Further technological innovation led to the use of phenylarsine oxide (PAO) agarose chromatography to enhance the Sec content of the recombinant TrxR preparation [12,13]. PAO-agarose works by binding vicinal Cys residues tightly [14]. Since full-length TrxR contains a Cys-Sec motif, a large fraction of the truncated enzyme can be excluded (though not exclusively) using this chromatographic step. The yield that this technology affords is impressive and the purity of this preparation is sufficient to enable the production of crystals of the Sec-containing enzyme [15].

Selenoproteins that do not contain a Sec residue near the C-terminus of the protein are less amenable to this technique. This is because the introduction of the *E. coli fdhF* SECIS element also contains coding information for amino acids within the stem-loop structure. Attempts have been made at producing recombinant selenoproteins where the UGA codon is near the 5' end of the gene. In the case of mouse methionine sulfoxide reductase B1, the selenoprotein was successfully produced, but the engineered protein contained mutations due the requirement of

the functional *E. coli* SECIS element in the middle of the open-reading frame [16]. Thus, this technique is best used for those selenoproteins where the Sec residue is found near the C-terminus, however a computer algorithm has been developed for the purpose of using an engineered SECIS element that minimizes mutations to the protein [17].

The heterologous expression of selenoproteins in cultured mammalian cells has met with less success, although for some complex mammalian selenoproteins, mammalian expression systems allow for post-translational modifications to occur. One such case is selenoprotein P (Sel P), the human version of which contains 10 Sec residues and is also glycosylated in several locations. Berry and coworkers were able to express Sel P in transiently transfected human epithelial kidney cells [18]. The produced protein was found to be glycosylated and the amount produced was sufficient for immunocharacterization. Gladyshev and coworkers developed an efficient system for expressing selenoproteins in mammalian cells that used a non-canonical SECIS element from *Toxoplasma gondii* that was found to be highly efficient at Sec insertion [19]. In this system, a plasmid was developed that contained this highly efficient SECIS element as well as the eukaryotic homolog of SelB – SECIS binding protein 2 (SBP2). While the overall yield of selenoprotein is increased in this system, it is still much lower than that of recombinant selenoproteins expressed in the *E. coli* system.

In contrast to these biotechnological approaches, a few groups (including my own) have focused on a chemical approach to the synthesis of selenoproteins. An early chemical method pioneered by Hilvert made use of the high reactivity of the active site serine (Ser) residue of subtilisin to make a synthetic selenoenzyme – selenosubtilisin [20]. The Ser residue was chemically converted to a Sec residue by first treating a solution of the enzyme with phenylmethanesulfonyl fluoride to create an activated sulfonylated Ser residue. Final conversion to Sec is then accomplished by addition of hydrogen selenide. This conversion occurred without side reactions and is only possible due to the exceptional chemical reactivity of this Ser residue in subtilisin. In theory, other serine proteases could also be converted to synthetic selenoenzymes using this method. The engineered selenosubtilisin possessed new enzymatic activities due to the presence of the newly created Sec residue. These activities included glutathione peroxidase activity and acyl transferase activity [20,21].

A second chemical method for the creation of synthetic selenoproteins is the technique native chemical ligations (NCL). This technique was first developed by Kent and coworkers and enables the chemical synthesis of moderately sized proteins by chemically joining two or more peptide fragments together [22]. One fragment contains a reactive α -thioester at the C-terminus and the other peptide has a N-terminal Cys residue (see Figure 1 for details). The two fragments are then able to react and form a stable amide bond. Two research groups led by Hilvert and van der Donk modified this technique to show that a Sec residue could take the place of a Cys residue to create a Sec-containing protein [25,26]. A recent, interesting example of using NCL to introduce a Sec residue into a Cys-containing protein in order to study the chemical advantages of Sec relative to Cys, is the creation of a synthetic seleno-glutaredoxin (Grx) [27]. Like thioredoxin, Grx contains a Cys-X-X-Cys motif. The Cys-X-X-Cys motif of Grx is responsible for reducing mixed disulfides between proteins and glutathione. Dawson and coworkers replaced the Cys residues of this motif to create Sec-X-X-Cys and Sec-X-X-Sec motifs. The Sec variants had lower redox potentials (-260 mV and -275 mV, respectively) than the wild type enzyme (-194 mV). Analysis of these redox equilibria with thioredoxin demonstrated that the lower redox potential of the Sec variants was due to the greater nucleophilicity of the selenol as compared to the thiol and not due to other factors such as greater leaving group ability.

My group used the related technique of intein-mediated peptide ligation (IPL and also referred to as expressed protein ligation – EPL) to make semisynthetic selenoproteins. The first example

of this was the replacement of Cys110 of RNase A with a Sec residue to create a semisynthetic seleno-RNase A enzyme [28]. The seleno-RNase A had similar activity as the WT enzyme, demonstrating that Sec for Cys substitution had no deleterious effects (see citation [29] for more details on seleno-RNase A). Protein semisynthesis has also been used to create Sec-containing mutants of azurin [30].

Success with the model enzyme RNase A led my group to attempt semisynthesis of naturally occurring selenoenzymes. Our approach divides the target selenoprotein into two modules, a non Sec-containing protein module produced as a recombinant protein in *E. coli* cells and a Sec-containing peptide module produced synthetically. This approach makes use of an engineered intein to catalyze the formation of a thioester at the junction of the intein and the target protein. The target protein can then be cleaved from the intein by thiolysis to produce a thioester-tagged target protein. The thioester group acts as a reactive electrophile, which can then be attacked by a peptide with a N-terminal Cys or Sec residue to form either a mixed thioester or mixed selenoester. Because the peptide contains a free terminal amino group, rearrangement occurs rapidly to form the amide bond (as shown in Figure 1), which is greatly favored thermodynamically. We have used this technique to produce two natural mammalian selenoproteins, TrxR [31] and methionine sulfoxide reductase B1. We review here the methods used to make these two selenoenzymes as well as new selenopeptide technology developed by my group that has further enhanced the study of the enzyme mechanism of TrxR.

Technological Difficulty Associated With Synthetic Sec-Containing Peptides

There is a great deal of interest in proteins and peptides that contain Sec, but the technology of selenopeptide chemistry has hampered the field for decades. First, biological scientists in the field do not have access to an inexpensive source of *L*-selenocystine, or a commercial source of a suitably protected Sec derivative for peptide synthesis. For a few years, the derivative Fmoc-Sec(Mob), was available from NovaBiochem for > \$2,000 per gram, and as far as this author is aware, the derivative is no longer commercially available. Two recent reviews have been published on methods for making *L*-selenocystine [32,33], so I will not elaborate further other than to say a common method (used by us) is to make *L*-selenocystine from elemental selenium and *L*- β -chloro-alanine. A second major problem is the compatibility of Sec with the various protecting groups that have been used for Cys. A common protecting group for Cys is the trityl (Trt –trimethylmethane) group. The Trt group is commonly used because it is easily removed with relatively mild acid such as trifluoroacetic acid (TFA), combined with appropriate scavengers. Its ease of removal is due to the high stability of the trityl cation. This property may make the Trt group incompatible with Sec due to the weakness of the C-Se bond, although there are no known reports of an attempted synthesis with the Sec(Trt) derivative. Peptide chemists have therefore chosen to use the benzyl (Bzl) [34-36] group and its derivatives, 4-methoxybenzyl (Mob) and 4-methylbenzyl (Meb) instead, due to the greater, relative instability of the benzyl cation, making C-Se bond scission much less likely with these types of protecting groups. While these protecting groups are compatible with both Boc and Fmoc chemistries and offer ease of synthesis to make the respective derivatives, they suffer from a lack of ability to be removed under mild conditions. The Bzl and Meb groups are usually removed with hydrofluoric acid (HF) or other very strong Lewis acids. The use of the Mob group with Sec, pioneered by Fuji and coworkers, has proven more useful due to much milder conditions that can be used to effect its removal [37]. For example, deprotection can be achieved using a mixture of DMSO/TFA (4-10% DMSO) [33]. My group has pioneered the use of a new reagent for the removal of the Mob group under very mild conditions [38,39]. This method makes use of 2,2'-dithiobis(5-nitropyridine) (DTNP) as a reagent to effect the removal of the Mob group. This reagent is a commercially available solid that dissolves in TFA and thus can be used in combination with other deprotection cocktails that are commonly used to remove other protecting groups from the peptide. Besides Cys, it is unreactive towards other peptide

nucleophiles, but as with other deprotection methods this method is not compatible with Trp residues because of a side reaction. A putative mechanism of deprotection is shown in Figure 2. This reagent can also be used to rapidly and efficiently form selenosulfide and diselenide bonds with concomitant deprotection in one step. This has been recently demonstrated to make Sec-containing analogs of apamin [40] as shown in Figure 3. The use of DTNP as a deprotection reagent for Sec(Mob) residues is the breakthrough that we needed to efficiently synthesize peptides to make semisynthetic selenoproteins and selenopeptide substrates to investigate the mechanism of TrxR.

Strategies for Creation of Semisynthetic Selenoproteins

There are 25 known genes in the human genome that encoded for selenoproteins [41]. The number of selenoproteins is slightly greater than 25 due to variants that arise through mRNA splicing. Although there are many barriers to heterologous selenoprotein production (as discussed in the introduction), a significant number of selenoproteins have the interesting property that the Sec residue is located near the C-terminus of the protein. This fact makes the 9 selenoproteins listed in Table 1 as capable of being made by semisynthesis using intein-mediated peptide ligation. In addition to the selenoproteins listed in Table 1, two other selenoproteins are candidates for study by chemical techniques. Selenoprotein W is a small protein of 87 amino acids that could be made by total chemical synthesis [42]. In addition, selenoprotein P has a cluster of 9 Sec residues very close to the C-terminus [43]. While the full-length selenoprotein P is unlikely to be made by chemical or semisynthetic techniques, the C-terminal Sec-rich module is a candidate to be studied by chemical techniques. It has been shown that selenoprotein P is a selenium transport protein [44,45], and while a receptor for selenoprotein P has been identified [46], the exact mechanism by which it delivers its Se atoms to intracellular targets is unknown. The chemical synthesis of Sec-containing peptides corresponding to the C-terminal module of selenoprotein P could aid in the study of this mechanism. Below we discuss the semisynthetic approach for production of two naturally occurring selenoenzymes, TrxR and methionine sulfoxide reductase B1 as well as other chemical approaches to the study of selenoenzyme mechanism.

TrxR is ideally suited for the modular semisynthetic strategy since the Sec residue is found as the penultimate residue in the enzyme [10]. In some organisms, such as *C. elegans* and *D. melanogaster*, the Sec residue has been replaced with the more common Cys residue [47,48]. Here and elsewhere, we denote the redox active tetrapeptide of high M_r TrxRs in the form X_{aa} -Cys₁-Cys₂/Sec₂- X_{aa} , where X_{aa} is any amino acid (usually Gly or Ser). This means that in this case, the Sec-containing, synthetic peptide will be relatively small. It is also advantageous that the Sec residue is part of a disordered, flexible tail region of the enzyme, so its removal does not affect the stability of the truncated protein. Using this strategy there are two different ways that a semisynthetic selenoprotein can be created. The first strategy is reminiscent of the RNase S/S-peptide system. In the early days of protein chemistry, it was shown that RNase A could be cleaved by subtilisin into two fragments: the S-peptide, which consists of residues 1-20 and the S-protein (RNase S), consisting of residues 21-124 and the two fragments could be separated by gel filtration chromatography [49]. Individually, the two fragments do not display ribonucleolytic activity, but when combined in a stoichiometry of 1:1, full enzymatic activity is restored. This type of peptide/truncated protein system works well because of the very tight binding of the S-peptide to the S-protein. This tight binding has led to the development of the so called S-tag system for use in affinity purification [50].

We used this identical approach to develop a non-covalent semisynthetic TrxR by creating a recombinant, truncated TrxR missing its last three amino acids Cys-Sec-Gly, and then adding a synthetic tetrapeptide containing the essential Sec residue (AcGly-Cys-Sec-Gly-OH) to the system to achieve very modest thioredoxin reductase activity. This strategy is outlined in Figure

4. As this system was not very robust [31], we proceeded to develop a semisynthetic TrxR in which we covalently linked the Cys-Sec-Gly tripeptide to a truncated form of TrxR.

A “covalent”, semisynthetic TrxR can be made by producing the truncated form of TrxR (missing the C-terminal tripeptide Cys-Sec-Gly) as an intein-fusion protein [31]. The intein system is marketed by New England Biolabs as the IMPACT[®] system. This system allows for the fusion protein to be produced in *E. coli* cells and as shown in Figure 5, allows for affinity purification (via chitin-agarose) through the use of a chitin binding domain that is attached to the intein. After lysing the cells, the supernatant is applied to the affinity column and then washed to remove impurities. Since TrxR is a flavoprotein, a large yellow band is visible on the top of the column. This yellow band can be removed from the column and placed in a separate vial where a buffered solution of peptide and exogenous thiol is added to the resin slurry. In principle, any small molecule thiol will do, but we like *N*-methyl mercaptoacetamide (NMA), because it is a water soluble, low pK_a thiol that can either attack the thioester at the TrxR-intein junction to form a thioester tagged TrxR protein, or it can keep the tripeptide in the reduced form. The reduced tripeptide can either attack the TrxR-intein thioester, or the nascent mixed NMA-TrxR thioester. Once the mixed thioester is formed, the free amino group on the N-terminus of the peptide can rapidly attack the carbonyl carbon to form a stable amide linkage. Once the amide linkage is formed, the enzyme will not undergo further modification. The semisynthetic TrxR produced in this way has very high catalytic activity (Figure 6) and compares well to both natively purified TrxR and recombinant forms of TrxR produced by the “Sel tag” method [12]. Using this method, we are able to introduce a number of mutations into TrxR to study structure function relationships [51]. Some of these mutations (summarized in Table 2) would be impossible to introduce using any other method, including the use of a genetically engineered mRNA transcript that contained a bacterial SECIS element. For example, we replaced the Cys₁-Sec₂ dyad of mammalian TrxR with a Sec₁-Sec₂ dyad. This mutation drastically reduced the catalytic rate of the enzyme, most likely because the rump enzyme containing the core catalytic elements of the enzyme, is unable to reduce a Se-Se bond. We also were able to replace the natural carboxylate group at the C-terminus with a carboxamide. Based on modeling studies it was predicted that a salt bridge formed between the C-terminal carboxylate group and a basic residue on the enzyme [52]. Using conventional site-directed mutagenesis techniques, replacement of the C-terminal glycine residue with any of the other 19 amino acids, would still result in a carboxylate group at the C-terminus. However, the use of protein semisynthesis allowed us to replace the COO⁻ group with a CONH₂ group, which is electrically neutral, but isosteric with the carboxylate group. The results in Table 2 show that this mutant actually has higher activity than the WT enzyme. While the results disfavor a salt bridge, a hydrogen bond is still possible between the C-terminal carboxylate and Arg351.

In addition to the mutants mentioned above, we were also able to insert Ala residues in between Cys₁ and Sec₂. This was done to probe the effect of ring size on the catalytic mechanism [51]. It is believed that a selenosulfide bond forms between the adjacent Cys₁ and Sec₂ residues, resulting in the formation of a rare 8-membered ring structure during the catalytic cycle [53]. Insertion of one and two residues in between Cys₁ and Sec₂ increases the ring size to 11 and 14 atoms, respectively. Surprisingly, these mutations had only a modest effect on the catalysis of thioredoxin, and this effect led us to hypothesize that the ring was relatively unimportant to the catalytic cycle of the mammalian enzyme. This point will be taken up in a later section.

Semisynthetic Sel R

In addition to our success with TrxR, we have also been able to produce methionine-*R*-sulfoxide reductase, or MsrB1, and also known as selenoprotein R (Sel R) as a semisynthetic selenoenzyme [54]. There are two other types of methionine-*R*-sulfoxide reductases, denoted

as MsrB2 and MsrB3, but these two subtypes have replaced the Sec residue with a conventional Cys residue. This replacement of Cys for Sec shows (like TrxR), a Sec residue is not chemically necessary to catalyze the reduction of methionine sulfoxide to methionine. Also like the TrxR system, it allows for the comparison of the chemistries of Cys and Sec in the same enzyme system.

Sel R is comprised of 116 amino acids with the Sec residue at position 95. Due to the relatively small size of Sel R and the favorable position of the Sec residue within the polypeptide chain, a semisynthetic strategy was envisioned that would allow for production of Sel R, as two distinct modules that could be joined together using peptide ligation chemistry. Production of residues 1-94 as an intein-fusion protein produced as a recombinant protein in *E. coli* cells allows for the formation of a reactive thioester group to be formed at F94. Residues 95-116 can then be produced as synthetic peptide with the Sec residue at the N-terminus. As shown in Figure 1, this allows for attack by the selenolate on the thioester to form a nascent selenoester. This selenoester, then rearranges to form the amide bond. The results of the ligation (and our strategy for making semisynthetic Sel R) are shown in Figure 7 as a 10% SDS-PAGE experiment. As is shown by the gel, upon addition of the peptide, a higher MW product is formed that corresponds to the MW of Sel R. The experiment also shows that ligation is incomplete because all of the 10 kDa thioester-tagged precursor is not consumed in the reaction. In our experience, when a Sec residue is at the ligation junction between the rump protein and the peptide (i.e. when the Sec residue is at the N-terminus of the attacking peptide) the ligation reactions are less efficient. This is likely to due to several factors; it is harder to keep the Sec-containing peptide reduced (in comparison to a Cys-peptide) and the nascent selenoester is more prone to alkaline hydrolysis. A second band of protein that is ~ 5 kDa in size is also present on the gel. This band appears only after the addition of a thiol cleavage reagent to the Sel R(1-94)-intein fusion protein. This additional band may be related to the novel 5 kDa selenoprotein form of Sel R that was recently described by Gladyshev and coworkers [55], however the form shown in the gel in Figure 7 must be different since it would obviously lack a Sec residue.

The unpurified semisynthetic Sel R enzyme produced by this technique had a specific activity of 37.5 nmol/min/mg protein (Gladyshev and Hondal, unpublished results). Purification to homogeneity was not attempted due to the small amount of protein produced in this experiment. We were interested in features of the protein architecture of Sel R that enables a Sec residue to replace a Cys residue in the MsrB2 and MsrB3 homologues. In the methionine sulfoxide reductases from MsrB2 and MsrB3, a highly conserved asparagine residue is found at position 97. However, in the Sec-containing MsrB1 (Sel R), a phenylalanine residue is found at this position. In a model of the active site based on the crystal structure of methionine sulfoxide reductase from *Neisseria gonorrhoeae*, Asn97 is very close to Cys95 [56]. Conservation of this geometry in Sel R would place Phe97 close to Sec95. We hypothesized that similar to sulfur-aromatic interactions found in proteins [57], the selenolate of Sec95 could be stabilized by a similar interaction [58]. To test this hypothesis, we constructed a semisynthetic Sel R in which Phe97 was replaced with pentafluorophenylalanine (pfPhe). In pfPhe, the distribution of the π electron cloud is completely different compared to Phe, in this case with negative charge in the plane of the ring and positive charge above and below the plane of the ring [59]. If an important interaction is being made between Sec and Phe, then this interaction should be disrupted in the mutant and a large decrease in activity should be observed. The semisynthetic Sel R that we created containing a pfPhe residue at position 97 had a specific activity of 1.7 nmol/min/mg of protein (Gladyshev and Hondal, unpublished results). This lower activity supports our hypothesis, but is not definitive. Nevertheless, this example demonstrates the power of semisynthesis for structure-function studies of selenoproteins, since it also allows for the incorporation of non-natural amino acids, in addition to allowing the incorporation of Sec for the production of the WT protein.

Use of Truncated TrxR and Peptide Substrates to Probe the Reaction Mechanism

The technique of peptide complementation discussed earlier is a useful technique to study the enzymatic reaction pathway of TrxR. In the case of TrxR, peptide complementation exploits the fortuitous and useful feature that the C-terminal selenosulfide motif acts as an “internal substrate” shuttle (analogous to GSSG in glutathione reductase) that is covalently linked to the enzyme. It is the reduced selenosulfide motif that then transfers electrons to the macromolecular substrate thioredoxin. If this shuttle system is physically disconnected from the enzyme by synthesizing the C-terminal redox motif as a synthetic peptide and using it as a substrate for the truncated enzyme, then the reduction and opening of this unique 8-membered ring motif, referred to us as the “ring opening” step, can be studied in great detail (see Figure 8) [60,61].

We have used this system to measure the rate of turnover of oxidized peptides either containing Sec or Cys at the second position (X in Figure 8) of the dyad. The results show that the Sec-containing peptide is turned over 300 fold faster than the Cys peptide using oxidized tetrapeptides as substrates. This result shows that the loss in activity upon Sec to Cys substitution in the mammalian enzyme occurs primarily in the ring opening step, and not the nucleophilic attack step as was commonly supposed. The difference in reactivity's increases further to ~8000 when oxidized octamer peptides are used as substrates. These experiments demonstrated the importance of Se in the thioldisulfide exchange step between N- and C-terminal redox centers.

Mammalian TrxR is not only unique because it is a Sec-containing protein, as a second unique feature is the 8-membered selenosulfide ring structure itself. A homologous 8-membered disulfide ring is found in TrxRs from *C. elegans* and *D. melanogaster*. There are only ~ 50 known instances of this structure from tens of thousands of deposited structures in the PDB [62,63]. Given the rarity of this structure in the PDB, an obvious question is why the enzyme would have evolved to use such a unique structural element. As part of the catalytic cycle, the enzyme must reduce the cyclic selenosulfide bond. We wanted to know how much less efficient the reduction of an *acyclic* selenosulfide bond would be in comparison. To answer this question, we constructed peptide substrates that contained either an 8-membered ring selenosulfide (using our chemistry described above), or an acyclic, interchain selenosulfide linking two peptide strands together (Figure 9). Using this strategy, the sequence of the peptide was conserved along with the selenosulfide bond, but the ring structure was disrupted. Using the ratio of the peptide turnover rate of these two substrates (ring/no ring) as a metric to determine the importance of the ring, we find that this ratio is rather small (~30). Performing the same experiment with either a cyclic 8-membered disulfide or acyclic disulfide showed that these two peptides were extremely poor substrates. This data informs us that the N-terminal active site containing the conserved sequence of CVNVGC has a great preference for cleaving a selenosulfide bond over that of a disulfide bond, with a preference for the selenosulfide bond within the context of an 8-membered ring structure [61].

This truncated TrxR/peptide system has also allowed us to investigate the preference of Sec for occupying the second position of the Cys₁-Sec₂ dyad. Again using synthetic peptide chemistry, we were able to switch the position of Sec to create a peptide with a Sec₁-Cys₂ dyad. This peptide is turned over 1100x slower than the peptide containing the correct Cys₁-Sec₂ oxidized dyad. We also did this experiment with the TrxR from *D. melanogaster* (DmTrxR), which normally contains a Cys₁-Cys₂ dyad. Using DmTrxR, we see that when we place Sec in position 1, the rate is slowed by a factor of 10, while it is increased by the same amount when Sec is placed in the second position (Figure 10). We have previously suggested the reason for this “seleno effect” is that the thiolate of Cys_{1C} must attack a more electronegative atom, resulting in a slower rate [61]. However, the electronegativities of sulfur and selenium are very similar [64] and an alternative reason for this decrease in rate might be that the sulfur atom of Cys₁ receives a proton from HisH⁺ upon either S–S or S–Se bond scission (depending

on the enzyme in question). Substitution of Se for S in this position would have the effect of slowing the rate because a selenolate is much less basic than a thiolate. Thus the Se atom could not accept the proton as readily as the S atom and this would result in a slower rate. If this is the explanation, this “seleno effect” would be completely analogous to the well described “thio effect” for phosphodiesterases in comparing the rate of hydrolysis of phosphates and thiophosphates [65,66].

Broad Substrate Specificity of Mammalian TrxR Explained by the Presence of a Selenium Atom or by Multiple Enzymatic Reaction Pathways?

The generally accepted reason for the very broad substrate specificity of the mammalian enzyme is thought to be the presence of a Sec residue in comparison to its Cys counterparts (We have catalogued the numerous statements in the literature attributing the broad substrate specificity of mammalian TrxR to the presence of the Sec residue in the supporting information of citation [72]). In addition to thioredoxin, macromolecular substrates include granulysin, protein disulfide isomerase (PDI), and Grx, while small molecule substrates include H_2O_2 , dithionitrobenzoic acid (DTNB), lipid hydroperoxides, vitamin K, ubiquinone, ninhydrin, alloxan, juglone, lipoic acid/lipoamide, dehydroascorbate, *S*-nitrosoglutathione, selenodiglutathione, selenite, and selenocystine [67]. Does the presence of a Sec residue actually account for all of the activities of mammalian TrxR? If one considers multiple enzymatic reaction pathways (with the corresponding evidence), and the types of bonds the CVNVGC active site can cleave, then it becomes clear that the presence of a Sec residue is not required for all of the reactions catalyzed by mammalian TrxR (Figure 11).

In addition to reducing the catalytic disulfide bond of thioredoxin, it is known that TrxR reduces a few other thioredoxin fold proteins such as PDI and Grx. For these macromolecular substrates, it is widely believed that the selenolate (Sec_2) is responsible for the initial nucleophilic attack on the disulfide of the macromolecule, forming a mixed selenosulfide between TrxR and the substrate (see citation [72] and references therein). While it has never been experimentally tested, it is believed that the next step in the mechanism is attack by the thiol from Cys₁. This would cause the release of product and result in the formation of the 8-membered selenosulfide ring. This ring must then be attacked by the thiolate of Cys_{1C}.

A point of disagreement in the mechanism concerns this step, with several groups presenting evidence that the Se atom is attacked [68,69,15], while our work has argued in favor of attack on the S atom from Cys₁ [60,61]. Our data using the truncated TrxR enzyme and synthetic peptide substrates indicated that Sec was required due to the superior leaving group ability of the selenolate in comparison to a thiolate, and on this basis we concluded that TrxR uses pathway 1 in Figure 11A. If on the other hand the enzyme uses pathway 2, then the selenolate would be the *second* leaving group in the ring opening step and not the first leaving group as proposed by us. If this turns out to be the case then the two different hypotheses can be reconciled by considering the proposal put forth by Brandt and Wessjohann [52,70]. They proposed that the mammalian enzyme uses Sec because the negatively charged selenolate was much better at stabilizing the positive charge on HisH^+ compared to that of a thiolate of Cys. However, we know that DmTrxR and other high M_r TrxRs catalyze the reaction without the need for Sec and there is a high amount of structural homology between the mammalian TrxR and DmTrxR [60]. The discovery that the pK_a of HisH^+ in the mouse mitochondrial TrxR is 7.8, while this value is 9.1 for HisH^+ from DmTrxR could explain the difference between the mammalian enzyme and its Cys-containing counterparts [61]. In both Sec- and Cys-containing TrxRs, once the 8-membered ring is reduced and opened, the residue in the second position of the dyad (X_2) should be ready once again to attack the disulfide bond of thioredoxin. In both classes of enzymes HisH^+ could be in an ion pair relationship with the atom from X_2 . The lower pK_a of the selenolate would then necessitate a lower pK_a of HisH^+ , while the higher

pK_a of the thiolate would require a higher pK_a on HisH^+ , which is the experimentally observed result. This explanation would favor pathway 2, but still be consistent with the role of the selenolate as a leaving group in the ring opening step. While this would be a satisfying way of reconciling the results reported in the field so far, more experiments are needed to clarify this point about the mechanism.

A second enzymatic pathway available to TrxR is termed by us as the “small molecule” mechanism (Figure 11B) because a number of small molecules can be reduced by the CVNVGC active site. The literature indicates that the CVNVGC active site of TrxR1 can reduce a number of different quinone compounds including ubiquinone and also DTNB to a lesser extent [71]. My group, working with TrxR2 (the mitochondrial enzyme), has shown that truncated TrxR2 can reduce DTNB to a large extent [60,61]. Because we were using a truncated TrxR/peptide system to investigate structure function relationships of the enzyme, we also discovered that lipoic acid is able to be efficiently reduced by the CVNVGC active site [72]. In addition, the Sec $\Rightarrow$ Cys mutant only has slightly less lipoic acid reductase activity than does the WT enzyme. This indicates that the presence of a Sec residue is not required for the reduction of lipoic acid. Thus at the moment, the small molecules DTNB, ubiquinone, and lipoic acid/lipoamide can be reduced by this pathway. Perhaps among the list of substrates for TrxR, others (and maybe some that are unknown) will also be discovered that can be reduced this pathway. We speculate that these small molecule substrates can compete with the Gly-Cys-Sec-Gly tetrapeptide for the tetrapeptide binding pocket and interaction with the N-terminal reaction center.

The use of lipoic acid as a substrate for the truncated enzyme demonstrates exactly what types of bonds can be cleaved by the CVNVGC reaction center as summarized in Table 3. As demonstrated by the data in the Table 3, the CVNVGC active site of the mammalian enzyme will not reduce unactivated -S-S- bonds such as those from “simple”, acyclic disulfides such as cystine. Acyclic disulfide bonds are able to be reduced if they are very electrophilic such as is the case with the disulfide bond of DTNB. In this case, the disulfide bond is highly polarized due to the presence of the 2-nitrobenzoate group. This polarization also leads to a very low pK_a of the thiol leaving group of the thionitrobenzoate anion (4.75) [73]. Thus, highly polar disulfides with low pK_a thiol leaving groups can be reduced by the CVNVGC active site. A third type of disulfide bond that can be reduced by the N-terminal reaction center is the disulfide bond of lipoic acid. The C-S-S-C dihedral angle of the 1,2-dithiolane is 35° and is thus highly strained, as it deviates 55° from typical disulfides, making this type of disulfide bond activated for cleavage [74,75]. Other types of cyclic disulfides such as a 1,2-dithiane (oxidized DTT), and an 8-membered vicinal disulfide ring from a Cys-Cys motif [61] are not substrates for the mammalian CVNVGC active site, demonstrating that the strain present in the 1,2-dithiolane of lipoic acid is the reason that its disulfide bond can be reduced.

In contrast, both cyclic and acyclic selenosulfide bonds can be reduced by the N-terminal reaction center of the mammalian enzyme as we have demonstrated through the use of our peptide substrates. We posit that selenosulfides are good substrates because they are polarized (compared to a typical disulfide bond) and a selenolate is a good leaving group. We note that a diselenide bond (-Se-Se-) such as the one from selenocystine is a poor substrate for the N-terminal reaction center. This is an interesting point as discussed below.

Another class of small molecule substrates for mammalian TrxR are themselves selenium containing compounds such as selenocystine, selenodiglutathione, and selenite [76,77]. TrxR may be responsible for reducing small molecule selenocompounds for use in selenium metabolic pathways. We have studied the reduction of selenocystine by TrxR as an example of this class of substrate. As the data in Table 4 indicates, there is only a small difference in the activities between the WT enzyme and the Sec $\Rightarrow$ Cys mutant TrxR. This is unlike the case

when macromolecular thioredoxin is used as the substrate as there is a 200-500 fold difference in activities between the wild type enzyme and the Cys mutant enzyme. This indicates that when selenocystine is a substrate, the enzyme utilizes a different enzymatic mechanism to effect its reduction. The data in Table 4 demonstrates that the enzyme must have an intact C-terminal reaction center to reduce selenocystine, but the reduction does not depend on the presence of a Sec residue. In addition, the truncated enzyme containing only the N-terminal reaction center reduces the -Se-Se- bond very slowly. However, the truncated mutant that contains a functional Cys₁ residue reduces the -Se-Se- bond at an intermediate rate (last entry of Table 4). These experiments have been very informative about the enzymatic mechanism. This data shows that the residue in the second position of the dyad must (be it Sec₂ from the WT enzyme, or Cys₂ from the mutant) be responsible for initiating nucleophilic attack on the substrate. The reason that the Cys₂-TrxR mutant enzyme has high activity with selenocystine must be two-fold. First, for very “soft” bonds such as a diselenide, the nucleophilicity of a Sec residue is not required for catalysis. Second, once a mixed diselenide bond forms between TrxR and the substrate (TrxR-Sec₂-Se-R), the S atom from Cys₁ can attack the Se atom from the substrate as shown in Figure 11C. This would mean that ring formation is “skipped” or bypassed in this mechanism. This step would lead to selenosulfide formation between the S atom of Cys₁ and the Se atom of the substrate, allowing the CVNVGC reaction center to reduce its preferred substrate, a selenosulfide bond as shown by the data in Table 3.

Thus we demonstrate that there are at least 3 distinctive enzymatic pathways for the mammalian TrxR to reduce a variety of substrates and these multiple pathways (and not the presence of Sec) help to explain the broad substrate specificity of the mammalian enzyme. The idea that TrxR uses multiple enzymatic pathways to catalyze the reduction of numerous substrates addresses an important point that was raised by Gromer and coworkers in their seminal hypothesis about the catalytic mechanism of mammalian TrxR [78]. Their hypothesis was that electrons are transferred from NADPH to the N-terminal redox center via the flavin. The N-terminal disulfide then reduced the C-terminal selenosulfide motif of the flexible tail (Figure 8), which then moved to a more exposed position to effect the reduction of substrates. While their hypothesis has been largely proven by experimental evidence, they also expected that a single mechanism could explain the broad substrate specificity of the enzyme as they stated:

“Clearly, any hypothesis for the catalytic mechanism of the enzyme must be able to explain this capability of the protein to reduce so many different substrates.”

In contrast to a single mechanism as a means of explaining the broad substrate specificity, our work explains substrate utilization in terms of multiple pathways as outlined above. This idea places new emphasis on the catalytic power of the N-terminal redox center. In addition to the pathways discussed above, there are likely other enzymatic reaction pathways used for substrates such as oxygen containing substrates such as dehydroascorbate and hydrogen peroxide [79,80]. This is an area under investigation in my laboratory. These other pathways have been discovered by studying the properties of the truncated enzyme in conjunction with synthetic peptide substrates.

Looking Forward

Protein semisynthesis has been shown to be an effective method for the production of mammalian selenoproteins. Until a universal biotechnological method is employed to solve the problem of heterologous production of selenoproteins, chemical approaches will continue to be used for the production of some selenoproteins (Table 1). Even if a solution is found, chemical methodologies will likely continue to be valuable tools for the elucidation of enzymatic mechanisms of selenoenzymes, and the placement of the Sec residue near the C-terminus of some of the selenoproteins in Table 1 may allow an analogous peptide

complementation approach to be used to study the function of the those proteins as has been the case for mammalian TrxR.

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Figure 1.

Method for introducing Sec into a protein by NCL. For this technique the α -thioester is generated chemically (A) and there are two techniques to do this. Using Boc chemistry, 3-thiopropionic acid is used as a linker to attach a peptide chain to the polymeric support via a thioester linkage [23]. If Fmoc chemistry is used, then usually a safety catch linker is used to connect the peptide to the solid support through a thioester linkage [24]. In both methods, a thiol (usually an aromatic thiol such as thiophenol) is used to cleave the peptide from the solid support to generate a C-terminal α -thioester-tagged peptide fragment (B). Once the peptide has been liberated from the solid support a synthetic peptide containing Sec (or Cys) at the N-terminus can then be added to solution of the thioester-tagged protein at neutral pH (C). The

Y group will attack the carbonyl of the thioester to form either a mixed selenoester (Y=Se) or a mixed thioester (Y=S) as in (**D**), which will rapidly rearrange to form a stable amide linkage. The result is a synthetic protein with a Sec residue incorporated at the C-terminus.

Figure 2.

Postulated mechanism of deprotection of a peptide Sec(Mob) derivative by DTNP. The Mob protected selenoether is still a good nucleophile and can attack the very polarized disulfide bond of DTNP and form a selenonium cation (middle). Water present in the cocktail can scavenge the Mob group leaving a 5-thionitropyridyl (5-Npys) derivative that can then be easily removed by thiolysis (right).

Figure 3.

Mechanism of thioselenide or diselenide formation in peptides using DTNP chemistry. A thioselenide bond can be formed in a peptide containing a Sec(Mob) residue and a Cys(Mob) residue ($\text{X}=\text{S}$) in a one step deprotection/oxidation procedure by adding a cocktail of TFA/DTNP/water to the peptide. Due to the high reactivity of the selenoether, the Se atom will attack the disulfide of DTNP to form a Sec(5-Npys) derivative, which then serves as a deprotection substrate for the neighboring Cys(Mob) residue. A similar mechanism is envisioned for diselenide formation when $\text{X} = \text{Se}$. In a peptide containing two Sec(Mob) residues and one Cys(Mob) residue, the diselenide is highly favored. The bold line represents the peptide backbone.

Figure 4.

Non-covalent semisynthetic TrxR/peptide system. The truncated TrxR can bind an oxidized peptide corresponding to its missing C-terminus and reduce it. The reduced Sec-containing peptide can then reduce the disulfide bond of Trx and start a new cycle.

Figure 5.

Method for producing semisynthetic TrxR by IPL. The cell lysate containing the recombinant TrxR-intein fusion protein is applied to the affinity resin and washed. The cleavage and ligation steps can be combined in one pot by adding a small molecule thiol and tripeptide Cys-Sec-Gly to the resin slurry. Upon washing the eluted protein has the tripeptide linked to it by an amide linkage and has high activity as shown in Figure 6.

Figure 6. Michaelis-Menten plot of semisynthetic Sec-containing mouse TrxR2 (filled squares) and the Sec $\Rightarrow$ Cys mutant produced by recombinant DNA technology (open circles). The mutant has 550 fold less activity than the semisynthetic enzyme using *E. coli* Trx as the substrate [51].

Figure 7.

Strategy for producing semisynthetic Sel R using IPL (top). The 10% SDS-PAGE experiment (bottom) shows the result of ligating the peptide fragment (red) with the thioester-tagged protein (blue). Lane 1 - molecular weight markers, Lane 2 – intein-Sel R fusion protein treated with β ME. There are two bands above and below the 6 kDa marker. The upper of these two bands is the truncated form of Sel R (residues 1-94). The lower band is an unidentified band that is related to Sel R. There are also three higher bands that correspond to uncleaved Sel R-intein fusion protein, Sel R fragment-intein fusion, and cleaved intein (starting in descending order from 17 kDa). Lane 3 – addition of Sec-peptide (red) to the same sample as in lane 2. A new band appears due to the ligation of the two fragments (shown by the arrow). Lanes 4 and 5 are additional fractions collected from the elution in lane 3. The results shown here are from unpublished data.

Figure 8.

A cartoon depicting the structure of mammalian TrxR (**A**). The C-terminal redox center is on a disordered, flexible tail of the enzyme and the selenosulfide motif essentially acts as an internal substrate for the N-terminal disulfide redox center. Note that X = **Se** or **S** in the diagram. The mechanism for the flow of electrons from NADPH to the substrate Trx is shown in (**B**) and the “ring opening” step is highlighted in red. We have used a truncated TrxR/peptide substrate system (**C**) to isolate the ring opening step from the overall reaction. The use of oxidized peptides as substrates for the truncated enzyme has demonstrated that the enzyme's ability to reduce the peptide is severely impaired when X = S. This implies that the rate limiting

step of the reaction is reduction of the oxidized C-terminal Gly-Cys-Sec-Gly motif and not reduction of the disulfide bond of thioredoxin.

A

B

Figure 9.

Comparison of the structure of cyclic (**A**) and acyclic (**B**) peptides used as substrates for the truncated TrxR. The experiment demonstrated that while the mammalian enzyme does prefer the cyclic Sec-peptide over the acyclic Sec-peptide by a factor of ~30, the presence of Sec in the peptide substrate is far more important than the ring structure itself as the sulfur versions of both peptides were extremely poor substrates for the truncated enzyme.

Figure 10.

Description of the “seleno effect” in mammalian TrxR. The truncated enzyme turns over the peptide 1100 fold faster when the Se atom is in the second position of the dyad (Se₂) as shown in (A) as compared to when the Se atom is in the first position of the dyad (Se₁) as shown in (B). This effect may be due to attack of Cys_{IC} on the “incorrect” atom in the dyad as we have previously suggested or may be due to the fact that the atom in the first position of the dyad receives a proton from HHIS⁺. This would make the reaction faster with a dyad consisting of S₁-Se₂ because S is more basic than Se.

A

B

Figure 11.

Description of the three different possible enzymatic pathways catalyzed by TrxR. **(A)** Macromolecular mechanism - TrxR uses this mechanism with macromolecular substrates like thioredoxin, PDI, and Grx. The key feature is that an 8-membered ring intermediate must be formed and then opened and this step is likely the rate limiting step. A point of controversy is whether the thiolate of **Cys_{IC}** attacks the Se atom (path 2) or the S atom (path 1) of the ring. **(B)** Small molecule mechanism - TrxR uses this mechanism with some small molecule substrates such as juglone, DTNB, and lipoic acid, plus perhaps other unknown substrates. In this mechanism the ring and the small molecule may be in competitive equilibrium for interacting with the **CVNVGC** active site. **(C)** Seleno-compound mechanism - TrxR uses this mechanism with selenocysteine and perhaps other selenium-containing molecules. Here, formation of the ring is skipped or “bypassed”. Note that **Cys_{IC}** is still faced with cleaving a S-Se bond, a known, preferred substrate. This step would be similar to the ring opening step from the macromolecular mechanism.

Table 1

Mammalian selenoproteins amenable to semisynthesis due to a C-terminal location of the Sec residue.

Selenoprotein	Position of Sec residue (total residues)	Length of Synthetic Sec-containing peptide
I	387 (397)	11
K	92 (94)	3
O	667 (669)	3
R	95 (116)	22
S	188 (189)	2
TrxR1 ^a	498 (499)	3
TrxR2 ^{a,b}	522 (523)	3
TGR ^a	655 (656)	3

^aThe synthetic peptide will begin with Cys and not Sec, increasing the length of the peptide by 1 one residue.

^bIncludes the length of the signal sequence.

Table 2

Kinetic parameters of WT and mutant mammalian TrxRs created by semisynthesis.^a

Semi	Enzymes		k_{cat}^b	K_m^c	%Pep In corp
	Synthetic				
		TR-Gly-Cys- Sec -Gly ₄₉₀ -C(=O)O ⁻	2220	68	91
		TR-Gly-Cys- Sec -Gly ₄₉₀ -C(=O)NH ₂	3010	42	63
		TR-Gly- Sec -Cys-Gly ₄₉₀ -C(=O)O ⁻	8	36	32
		TR-Gly- Sec -Cys-Gly ₄₉₀ -C(=O)O ⁻	1	65	10
		TR-Gly- Sec -Cys-Gly ₄₉₀ -C(=O)O ⁻	350	21	100

Semi	Synthetic	Enzymes			
			k_{cat}^b	K_m^c	%Pep Incorp

^aData taken from [51]. *E. coli* Trx was used as the substrates for these experiments.

^bUnits are (min⁻¹)

^cUnits are (μM)

Table 3

Types of bonds reduced by the N-terminal reaction center (CVNVGC) of mammalian TrxR.

Compound		Substrate?	Reason
n=1	Yes ^a	Strained ring of dithiolane
	n=2	No ^a	Lack of ring strain in dithiane
X = S	No ^b	Poor leaving group ability of S relative to Se
	X = Se	Yes ^b	Superior leaving group ability of Se
X = S, R = Cys	Yes ^c	In both cases, a highly polarized disulfide bond and excellent leaving group ability of TNB anion.
	X = S, R = 2-nitrobenzoate	Yes ^b
X = S, Y = S	No ^a	Inactivated disulfide bond
	X = S, Y = Se	Yes ^a	Activated selenosulfide bond
	X = Se, Y = Se	No ^a	?

Reported in citation [72].

Reported in citation [61].

These results are unpublished [Hondal and coworkers].

Table 4

Activity of WT and mutant TrxR2 enzymes toward selenocystine and cystine^a.

Enzyme-tetrapeptide ^b	Activity (mol NADPH/min/mol TR)	
	Selenocystine (-Se-Se-)	Cystine (-S-S-)
mTrxR-GCUG	295 ± 21	3 ± 1
mTrxR-GCCG	79 ± 3.4	0.9 ± 0.04
mTrxRΔ8	0.6 ± 0.05	0.4 ± 0.05
mTrxR-GC	12.5 ± 2	ND

^a Performed at pH 7.0 and 91 μM substrate concentration at pH 7.0. The TrxR used here is the mouse TrxR2. These results were reported in citation [72].

^b The enzymes are abbreviated as mTrxR-Gly-Cys-Sec-Gly to signify the sequence of the C-terminal redox active tetrapeptide. The changes to the tetrapeptide are as indicated and Δ8 signifies that the C-terminal 8 amino acids have been removed.