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INVESTIGATION INTO THE ROLE OF THE C-TERMINAL VICINAL CYSTEINE RESIDUES IN HIGH $M_r$ THIOREDOXIN REDUCTASES

A Thesis Presented

by

Brian M. Lacey

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Masters of Science
Specializing in Biochemistry

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Abstract

Mammalian thioredoxin reductase (TR) contains the rare amino acid selenocysteine (Sec), which is essential for the enzyme’s catalytic activity. Substitution of the catalytic Sec residue for a cysteine (Cys) residue, results in a drop in $k_{cat}$ of 100-fold. Homologous high molecular weight TRs from other eukaryotes such as D. melanogaster and C. elegans, have naturally evolved a Sec to Cys substitution in their active sites and these enzymes function with high catalytic activity without the need for a Sec residue. Thus, various TRs can catalyze an identical reaction with either a Cys or Sec residue. A natural assumption in the field has always been that the lower nucleophilicity of a Cys thiol, relative to the selenol of Sec, is the reason for the much lower activity of the mammalian Cys-containing mutant. However, here I provide an alternative explanation. High $M_r$ TRs contain either a Cys-Cys or Cys-Sec dyad that forms an eight-membered ring in the oxidized state during the redox cycle of the enzyme. These eight-membered ring structures are rare in protein structures, presumably due to the strain induced in the intervening peptide bond between the Cys residues. Here I take a “chemical approach” to studying the enzyme mechanism of TR by breaking it into two pieces. This approach is possible because of TR’s structural and mechanistic similarity to glutathione reductase (GR). In comparison to GR, TR contains an additional thiol-disulfide exchange step resulting from the presence of a sixteen amino acid C-terminal extension containing either a vicinal disulfide bond or vicinal selenylsulfide bond. This additional thiol-disulfide exchange step is in the form of the reduction and opening of the eight-membered ring motif. I have constructed a truncated version of the enzyme lacking the amino acid sequence possessing the ring motif so that I could isolate this ring-opening step from the rest of the catalytic cycle by using peptide disulfides/selenylsulfides as substrates. The results of this study using peptide substrates show that the ring opening step is the step of the catalytic cycle that is most effected by Sec to Cys substitution because the higher $pK_a$ of the Cys thiolate in comparison to the Sec selenolate means that the Cys residue must be protonated in this step.
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Dedications

I would like to dedicate this dissertation to my parents Eugene and Debbie for providing me with the love, support, and moral courage to follow.

Additionally, I would like to thank Shaina for saving me when I needed to be saved and for all of her love and support throughout this journey.
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Chapter 1

Introduction to Thioredoxin Reductase

Introduction

Redox Systems
Oxygen is the molecule of aerobic life. Life uses the energy in molecular oxygen to drive ATP synthesis which in turn fuels all the other critical chemical reactions occurring in an organism. A side effect of oxygen is oxidation and formation of reactive oxygen species. The cell must deal with these oxidation products or havoc will result upon the cellular machinery. To maintain cellular redox homeostasis two systems have evolved: the thioredoxin system and the glutaredoxin system (1). Both of these two systems use gradients of electron potential to act as an electron flow pathway to ultimately reduce a target substrate. In each system, NADPH, acts as the primary hydrogen donor because its redox potential is extremely low (2).

The glutaredoxin system
The glutaredoxin system consists of glutaredoxin (grx), glutathione, NADPH, and glutathione reductase. In this system electrons are transferred from NADPH, through glutathione reductase, which reduces its substrate oxidized diglutathione (GSSG) to two molecules of glutathione (GSH), which then in turn act upon grx yielding its reduced form. Grx functions to deglutathionylate proteins, similarly as phosphatases dephosphorylate proteins (3). This system acts to maintain high levels of reduced glutathione which acts as a thio-disulfide redox buffer (4). The pool of glutathione is
kept almost exclusively reduced, with ratios of up to 200:1 reported compared to the oxidized (5).

*The Thioredoxin System*

In addition to the glutaredoxin system, the thioredoxin system functions as a critical antioxidant system that is also responsible for maintaining the reduced cellular environment. This system is composed of two oxidoreductase enzymes thioredoxin (Trx) and thioredoxin reductase (TR) as well as the reducing cofactor NADPH. (6)

Trx is a small protein of approximately 12 kDa that is responsible for reducing many intracellular cellular targets, and hence must be constantly cycled back to the reduced state by TR (6-8). The Trx active site consists of a conserved CXXC motif (9) that when oxidized forms a disulfide bond. The reduced, dithiol form of Trx is responsible for being the hydrogen donor of many substrates, but was first described for its role in the enzymatic synthesis of deoxyribonucleotides by ribonucleotide reductase in *E.coli* (10). It has since been described to be involved in many reactions in which disulfide bond formation results, such as with methionine sulfoxide reductase (11) and peroxiredoxins (12).

The redox regulation of various proteins by the Trx system has also been recognized. For instance, Trx must reduce a single cysteinyl residue, to allow for the association of NF-κB subunit p50 to its DNA target sequence (13). Trx catalyzed reduction also results in the activation of many transcription factors, with some examples consisting of the tumor suppressor p53 (14), the estrogen receptor (15), and glucocorticoid receptor (16).
Thioredoxin Reductase

The buildup of oxidized Trx is the consequence of the reduction of cellular disulfides. For Trx to maintain its function, Thioredoxin reductase (TR) (EC 1.6.4.5) an essential part of the thioredoxin system, must act as an equalizing factor to maintain Trx in its reduced state, to prevent the upsurge of the oxidized Trx pool (17). TR is a member of the family of pyridine nucleotide-disulfide reductases, which function to maintain cellular redox homeostasis (18). Other enzymes in this family include glutathione reductase, lipoamide dehydrogenase, and mercuric ion reductase, which are all highly conserved structurally. Members of this family are all homodimers that accept reducing equivalents, usually in the form of NADPH, which donates a hydride to an enzyme bound cofactor (FAD), which in turn forms a charge-transfer complex with an enzyme disulfide and then transfers the electrons to the target substrate (19) (Figure 1A).

Two forms of TR have evolved in nature, low and high molecular ratio (Mr) enzymes. Low Mr TRs (35 kDa) are found in prokaryotes, archaea and lower eukaryotes, while high Mr TRs (55 kDa) are found in higher eukaryotes. Both forms catalyze the identical reaction, though different catalytic mechanisms have evolved for each. (20, 21).

The mechanism of the low Mr TR, from *E. coli* (EcTR) has been reviewed by Williams (22). The general mechanism of this enzyme is the same as the other family members such as glutathione reductase (GR) (Figure 1B). However there are several distinct differences between them. In addition to the noticeable differences in the Mr between the two classes, the crystal structure indicates that the mechanism requires a large conformational change upon binding of NADPH to the NADPH binding domain. A
rotation of 66° is necessary to orient the pyridinium ring in proximity of the FAD for electron transfer (23). This conformation change is not observed in the high Mr TRs.

The high Mr TRs contain an additional C-terminal redox center compare to EcTR and other family members. This C-terminal redox center contains a dithiol/disulfide redox pair, which first accepts two electrons from the N-terminal dithiol/disulfide, and it is this C-terminal motif that catalyzes reduction of the target substrate Trx (21) (Figure 1C). This C-terminal redox center varies from species to species.

This reaction scheme is highly conserved among this family of enzymes, with GR and TR sharing the highest similarity. The functional enzymatic unit is a homodimer. NADPH binds to the NADPH binding domain transferring electrons in the form of a hydride to a FAD cofactor non-covalently associated to the enzyme. The isoalloxazine ring of the flavin interacts with a conserved N-terminal redox center of sequence C_{1C}VNVGC_{CT}. This redox center must be reduced and results in the formation of a charge-transfer complex with the FAD and the C-terminal Cys residue (abbreviated as Cys_{CT}) from the N-terminal redox center. The N-terminal interchange Cys_{IC} of the redox center then forms a thiolate which is capable of nucleophilically attacking an incoming disulfide substrate and forming a mixed disulfide, causing release of the first leaving group. During this step a conserved acid/base histidine, from the opposing subunit, stabilizes the leaving group by donation of a proton. The Cys_{CT} residue then completes the reaction by resolving the mixed disulfide, resulting in release of the substrate and reoxidation of the N-terminal redox center. For GR the disulfide substrate is GSSG, and results in the release of two GSH and a recycled enzyme. In the case of
TR, this same N-terminal disulfide/dithiol redox center transfers its reducing equivalents to a C-terminal dithiol/disulfide redox center on the opposite subunit, which can be considered analogous to the disulfide substrate, except that it is tethered to the enzyme. The “release” of a reduced C-terminus results in the continuation of its catalytic cycle through an additional step, reduction of its native substrate Trx before it is recycled.

High \( M_r \) TRs have two particularly interesting features concerning their C-terminal redox center. In some higher eukaryotes, including mammals, it contains selenium in the form of selenocysteine (Sec, U) – the 21\(^{st}\) amino acid as the penultimate amino acid (24), while other eukaryotic homologs have replaced the Sec residue with a conventional cysteine (Cys) residue. The second interesting feature concerning their C-terminal redox center, involves the Sec or Cys residue as part of a vicinal selenylsulfide or a rare vicinal disulfide bridge as part of their catalytic cycles (25, 26). The next section will discuss these unique features.

**Selenium as Selenocysteine**

Selenium is an essential trace element present in all kingdoms of life. In its elemental form selenium is toxic so it must be converted to low MW compounds such as selenodiglutathione, methylselenol, selenide, selenomethionine and Sec by plants (27).

The most biologically active form of selenium is in the form of Sec, a Cys analog. At physiological pH, these two amino acids have differing ionization states. Sec is ionized and more highly reactive than Cys, while Cys is normally more stable and protonated in its thiol form. This is due to Sec’s lower \( pK_a \) of 5.3 compared to Cys (8.2) (28). Sec becomes biologically active upon its incorporation into selenoproteins. The human
selenoproteome consists of 25 known selenoproteins (29). Of these selenoproteins, the most characterized are glutathione peroxidases (GPx), iodothyronine deiodinases (DIO), selenophosphate synthethescs 2 (SPS2), and TR, and all have oxidoreductase functions (30).

Sec incorporation was first described being coded for by the opal codon UGA in mouse glutathione peroxidase (31). Sec is synthesized through a serine intermediate on its own unique tRNA, named tRNA[^Ser]Sec (32, 33). The tRNA[^Ser]Sec is loaded with serine via seryl-tRNA synthase. The loaded seryl-tRNA[^Ser]Sec is then phosphorylated by phosphoseryl tRNA kinase resulting in a phosphoseryl-tRNA[^Ser]Sec. To finish the synthesis of Sec, Sec synthase reacts with the activated selenium donor, monoselenophosphate, and the phosphoseryl-tRNA[^Ser]Sec to yield Sec-tRNA[^Ser]Sec (34).

To decode the UGA codon as a sense codon more than just the Sec-tRNA[^Ser]Sec is required. A secondary RNA stem-loop structure termed the Sec Insertion Sequence (SECIS) element must be present, otherwise translation will result in termination. In eukaryotes, the SECIS element is locate within the 3′-untranslated region (UTR) of the mRNA (35). Additionally, specific Sec-decoding protein factors are needed such as Sec-specific elongation factor (mSelB/eEFSec) (36, 37) and the SECIS-binding protein (SBP2) (38, 39) for UGA decoding. The Sec-decoding apparatus recently had some new additions, three additional proteins, the soluble liver antigen protein (SLA), Seep43 (the 43 kDa RNA-binding protein) (40), and the ribosomal protein L30 (41).

Selenocompounds have shown effectiveness in the treatment and prevention of cancers leading to the search for therapeutic agents. TR has been proposed as a good
anticancer target because this enzyme is necessary for cancer cell proliferation and has been shown to be up-regulated in some cancer cell lines. The development of TR inhibitors is an area of ongoing research, with several examples (reviewed in (17)) including nitrosoureas (42), cisplatin (43), the antitumor quinoid compounds such as diaziquone and doxorubicin (44, 45). These compounds are all believed to interact with the C-terminal active site thiol or selenol, and disrupt TR’s ability to recycle Trx, leading to an oxidized cellular milieu.

*Vicinal Disulfide Bonds*

The other unique characteristic of the high $M_r$ TR involves formation of either a vicinal selenylsulfide or disulfide bridge on its C-terminus. In the Brookhaven Protein Data Bank (PDB), a disulfide bond between neighboring Cys residues, $(i, i + 1)$, is a rare occurrence. In a study by Pongor and coworkers, there were only 32 occurrences of this type of disulfide bond from 28,000 deposited structures (46). The reason for the infrequency of this type of disulfide bond is most likely due to the strain imparted on the intervening peptide bond between adjacent half-cystinyl residues (47). This strain is due to the existence of an eight-membered ring motif which contains a central amide bond possessing partial double bond character. The ring strain imparts flexional torsion onto the region of a peptide or protein where it is found in nature (48, 49). For example, NMR studies have indicated that the most flexible part of human and bass hepcidin, as well as the Janus-faced atracotoxin (J-ACTX) is the region of the protein where this ring is found (50-52).
This eight-membered ring motif almost always exists as part of a type VIII β-turn, helping to stabilize this type of high energy turn structure (46). It is also found in various enzymes such as methanol dehydrogenase, mercuric ion reductase, as well as TR (53-55). In these systems, the Cys-Cys dyad cycles between reduced and oxidized states as part of a redox cycle. In addition to a catalytic role for this motif, it has been proposed that this flexible ring is part of a conformational switch, controlling active and inactive states of the protein. This is apparently the case in the nicotinic acetylcholine receptor where it has been proposed that switching between cis and trans forms of the ring regulates the on/off state of the receptor (49, 56). A regulatory role for this motif has also been proposed for cytochrome C oxidase from Utricularia (57).

The sequences of the high M, TRs C-terminal redox center motif varies from species to species. The mammalian enzyme is of particular interest because its C-terminal redox center contains Sec as part of a conserved tetrapeptide GCUG motif (24, 58). The Sec residue of this motif has been shown to be essential for function and is thought to impart the enzyme with broad substrate specificity other than its natural target of Trx including hydrogen peroxidase activity (59).

Many TRs lack Sec, and are able to catalyze the reduction of the catalytic disulfide bond of Trx with a Cys residue in place of Sec. For example, in the case of the TR from Drosophila melanogaster (DmTR), the enzyme contains a C-terminal SCCS motif and this enzyme can reduce its cognate Trx with high efficiency, thus demonstrating that the reaction can be catalyzed without the need of a selenium atom (25). It has recently been proposed that the reason that DmTR can function with high
catalytic efficiency using Cys in place of Sec is that the flanking serine residues increase the nucleophilicity of the thiol-nucleophile by mediating proton transfer from the attacking thiolate to the leaving group sulfur atom of the substrate (60). Mutation of the N-terminal serine residue to a glycine residue had little effect on catalytic parameters, while mutation of the C-terminal serine to a glycine residue decreased $k_{\text{cat}}$ by two-thirds. Thus it seems that for DmTR, the C-terminal serine residue plays some role in catalysis.

However, not all TRs in which the Sec residue has been replaced with Cys contain flanking serine residues. The TR from *Anopheles gambiae* (AgTR) contains an N-terminal threonine, and in the case of the mitochondrial TR from *C. elegans* (CeTR2) the flanking residues are glycine (61, 62).

**Proposal /Specific Aim**

With the unique features of TR C-terminus being variable between species I am interested in studying the role of the vicinal Cys disulfide eight-membered ring motif. Since this structure has been implicated in being a rare structure in nature, I want to evaluate the importance of this eight-membered structure in the catalysis of TR. The residues that compose the ring are known to be essential for activity, however to date no one has evaluated the role of the ring structure in the catalytic cycle, therefore I want to see if the structure itself is necessary for catalysis.

It is known that Sec is essential for the mammalian TR enzymes. However is has also been demonstrated that other high eukaryotes, *D. melanogaster* and *P. falcifarum*, utilized Cys and have comparable catalytic activities with the Sec containing counter
parts. A second question I evaluate in the thesis is, why do some TRs utilize Sec while other TRs are just as capable utilizing Cys?

Lastly, I intend to evaluate the answers to both of these questions to determine if they are mutually exclusive or directly related.

It is my hypothesis, that for the enzymes containing a Cys-Cys dyad (CeTR2 and DmTR), the presence of an eight-membered ring will be important to catalysis, while for the Sec-containing mouse enzyme (mTR3) the ring structure will be of less importance. In the active site of both TR and GR there is a conserved histidine residue that is essential for activity (see Figure 3). During the catalytic cycle of GR, this histidine must donate a proton to the first leaving group, GSH I. This part of the reaction is analogous with the ring opening step of TR, where the Cys$_2$ or Sec$_2$ position of the dyad would act as the first leaving group. I believe that for the Cys containing TRs, the ring structure is essential for aligning the leaving group with this conserved histidine, to allow for proton donation and stabilization of the leaving group thiolate. Conversely, the Sec residue of the Sec class of TRs does not need to be protonated due to its low $pK_a$ leaving group, thus making the eight-member ring less important.

Before I could evaluate these questions I wanted to characterize the mitochondrial C. elegans TR (CeTR2) because its C-terminal sequence of GCCCG is similar to the mTR3 sequence of GCUG, which contains Sec and identical to mTR3 U to C mutant that lacked significant activity (59). This sequence is unique compared to other examples of TR from the literature that evolved using Cys in place of Sec such as the DmTR and PfTR enzymes. Of these two examples, DmTR has a C-terminal sequence of SCCS,
which contain the vicinal disulfide motif without the use of Sec. It has been proposed that the flanking serine residues activate the Cys residues making them more reactive (60). Of the other example, PfTR has a C-terminal sequence of GCGGGKGCG (63). This sequence lacks the flanking serine residue, but it also lacks the vicinal Cys motif with insertion of four amino acids between the reactive redox center. The cloning and characterization of the CeTR2 enzyme as intein mediated fusion protein will allow for us to examine it for its ability to reduce Trx. This additional example of a Cys TR and comparison of its activity with the other Cys TRs and the Sec TRs will validate or disprove the proposal (mentioned earlier) put forth by Gromer in regards to the role of flanking serine residues enhancing activity (60). Additionally, mutations that mimic the DmTR motif, producing a CeTR2-SCCS construct will determine if the flanking serine residues are necessary for activity or enhancing activity.

To evaluate the importance of the eight-membered ring structure in the catalysis of TR, I propose to disrupt the WT conformation of the C-terminal ring. Mutagenesis will be used to insert either one or two alanine residues between the vicinal Cys residues. These insertions will enlarge the ring by three and six atoms producing eleven and fourteen-membered rings, respectively. The effect of these insertions will be examined by assaying these mutant enzyme constructs for TR activity to determine how important the eight-membered ring is to catalysis, and if the larger membered, non-vicinal disulfide rings can catalyze the reaction.

To evaluate why some TRs utilized Sec while others TRs utilize Cys, a peptide complementation technique can be utilized in which truncated TRs lacking the C-
terminal redox motif from both Sec and Cys TR classes, are assayed against peptide substrates mimicking their C-terminus (Figure 4). The comparison of activity of WT (Cys or Sec) cyclic peptides, which maintain specific conformations, with their corresponding acyclic peptides, which have no rigid conformation because the peptide bond between the vicinal Cys or Cys/Sec has been severed, should reveal details about the importance of the ring structure. A similar comparison of activities between acyclic peptides containing Sec or Cys in the penultimate position, will allow an evaluation of the effect the leaving group has on the catalytic reaction. Also an acyclic mixed disulfide of the peptide substrate conjugated to the highly reactive, low pKₐ TNB (5-thio-2-nitrobenzoate) anion, acts as a Sec containing peptide analog. A final “switch” peptide, in which the Sec and Cys residues have been swapped resulting in Cys in the penultimate position, will be assayed. This peptide will result in information regarding, whether Sec or Cys is the atom attacked by the interchange CysIC residue. Results regarding these assays will allow us to propose a model for the mechanistic differences between the two classes of high Mr TRs.
**Figure 1: Conserved enzymatic reaction scheme**

A. Conserved enzymatic reaction scheme carried out by family of pyridine nucleotide-disulfide reductases. NADPH, which donates a hydride to an enzyme bound cofactor (FAD), which in turn forms a charge-transfer complex with an enzyme disulfide and then transfers the electrons to the target substrate disulfide.  

B. For the low $M_r$ TR, the substrate disulfide is Trx.  

C. For the high $M_r$ TRs, the conserved enzyme disulfide reduces an addition enzyme disulfide redox center (from the opposing subunit of homodimer, denoted by prime (') symbol), which which reduces the Trx disulfide substrate.
Figure 2: Peptide Complementation
In this technique, the C-terminus that consists of the additional enzyme disulfide redox center is removed, creating a truncated TR enzyme. This truncated TR can reduce, oxidized disulfide peptides (see Figure 4) that mimic the C-terminus, allowing for the kinetic investigation of this mechanism. Arrows denote flow of electrons through enzyme.
Figure 3: Conserved Active Site Histidine

A. Following reduction of the conserved enzyme disulfide, Cys_{IC} thiolate attacks the disulfide substrate (oxidized glutathione for GR) or the additional enzyme disulfide (C-terminal vicinal disulfide of TR), resulting in the formation of a mixed disulfide between Cys_{IC} and the release of the leaving group thiolate. The leaving group thiolate must be protonated by the adjacent His residue, and can stably leave. Resolution of the enzyme disulfide is initiated by attack of Cys_{CT}, and release of the second thiol. (Figure adapted from Ref. (64))

B. Alignment of the tetrapeptide SCCS(ox) in the structure of TR from Drosophila. The tetrapeptide structures were determined by NMR and placed in the active site of TR in accordance to the position of Glutathione I (the interchange position) in the GR structure (PDB 1GRA). Shown are the FAD cofactor (dark red) and the helical loop of the conserved active site sequence CIC-VNVC_CT, which are components of chain A. Also shown is Arg473’ from the B chain as well as His464’ and Glu469’, which are analogous to His467’ and Glu472’ in GR. The oxidized C-terminal disulfide of the B chain is reduced by the FAD-associated disulfide of the A chain during the enzymatic cycle. The residues from TR are in gray, oxidized glutathione is in purple, the tetrapeptide is in green. Cys57 (Cys_{IC}) of DmTR is in position for interchange with glutathione I and the Cys489’ of the SCCS(ox) tetrapeptide, which is indicated by the orange dashed line. Glutathione II is in position for protonation from His464’ as is Cys490’ of the SCCS(ox) tetrapeptide as indicated by the red dashed line. Only the cysteines for the SCCS(ox) peptide and GSSG are shown for simplicity. The prime designation for residue numbers indicates the B chain of the TR homodimer. (Figure adapted from Ref. (65))
Figure 4: Peptide substrates constructed for this study.
A.) Typical WT cyclic octamer peptide, (X = S or Se atom of Cys or Sec, respectively), that must reduced during TR’s catalytic cycle.  B.) Acyclic octamer peptide. The peptide bond between the vicinal Cys or Cys/Sec has been disconnected, thus preventing formation of the cyclic structure.  C.) Acyclic hexamer, Cys₁ forms a mixed disulfide with the thiol of the highly reactive TNB anion, referred to as Pep-TNB in the text.  D.) Cyclic “switch” mutant. The typical Cys₁-Sec₂ dyad of the eight-membered ring has been “switched”, resulting in a Sec₁-Cys₂ dyad.
Chapter 2

Characterization Of Mitochondrial Thioredoxin Reductase From C. elegans

Introduction

The mitochondrial C. elegans thioredoxin reductase (CeTR2) contains the C-terminal motif of GCCG (62, 66), lacking the Sec residue so crucial to the mammalian enzyme (59). It has recently been demonstrated that other higher eukaryotic species contain TR lacking Sec (25, 60, 61, 67), but retain high catalytic activities. In the case of the DmTR, where its C-terminal motif consists of sequence SCCS, it was postulated that the flanking serine residues adjacent to the vicinal Cys’s aid in thiolate formation (60). It is hypothesized that this serine activation, is the cause of the resulting high catalytic activity. To examine this hypothesis, here I report the cloning, purification and enzymatic characterization of CeTR2. The recombinant CeTR2 was expressed as an intein mediated fusion protein and characterized for TR activity. I also report mutations to this C-terminal motif to SCCS and GCCS, to experimentally determine the effect flanking serine residues would have on the catalytic rate of Trx reduction to evaluate if they contribute to the rate as hypothesized by Gromer (60).

Methods

 Mutagenesis and transformation.

The coding sequence for CeTR2 was amplified from an EST clone purchased from Open Biosystems (clone ID ZK637.10) using PCR. The upstream primer had the sequence 5’-AACAGACATATGTTTCATCAAATAAATTTGATCTGATTG-3’, and
the downstream primer was of sequence 5′-ACAGCCGCTCTTCAGCATCCACAGCATCCCTGAGTT-3′. The PCR was performed on a Perkin-Elmer GeneAmp PCR System 2400. The PCR (100 µL) contained 100 pg of template DNA (pDONR201, containing the coding sequence), 50 pmol of each primer, 2 U of Vent DNA polymerase, 200 µM of each dNTP, in a buffer containing 20 mM Tris–HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, and 0.1% Triton X-100, pH 8.8. The steps in the PCR were as follows: 96 °C (initial denaturation) for 2 min followed by 25 cycles of 96 °C (45 s), 55 °C, (30 s), and 72 °C (3 min). The amplification reaction was monitored by analytical agarose gel electrophoresis (staining with ethidium bromide). The DNA from the PCR was then purified using the Qiagen QIAquick PCR Purification Kit. The PCR product was then treated with restriction enzymes NdeI and SapI for 2 h at 37 °C in 50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9, in a total volume of 50 µL. The digest was then purified using the Qiagen QIAquick PCR Purification Kit and the purified DNA was used in a ligation reaction to subclone the amplified gene into plasmid pTYB1. Two hundred nanograms of plasmid pTYB1 and 400 ng of PCR product were incubated with 400 U T4 DNA ligase at 16 °C overnight. The ligase was inactivated by heating the reaction at 70 °C for 30 min. The ligation reaction was then incubated at 37 °C with EcoRI for 2 h to enhance the amount of positive clones because EcoRI will only cut plasmids that have not been ligated with the PCR insert. The resulting plasmid (pCETR2) encodes a fusion protein consisting of CeTR2, VMA1 intein, and a chitin-binding domain. Escherichia coli DH5α cells made competent by CaCl₂ were transformed with 20 ng of pCETR2 (68). The cells were plated
on LB-agar containing ampicillin (200 µg/mL). Positive clones were screened by restriction analysis using HindIII and NdeI, and analyzed by analytical agarose gel electrophoresis. Several positive clones were then selected for DNA sequencing to verify the wild-type coding sequence. All sequencing was performed by the University of Vermont DNA Sequencing Facility using an ABI 3100-Avant Genetic Analyzer.

Two mutant *C. elegans* TRs were also constructed using PCR using the same conditions listed above except that downstream primer 5’-ACAGCCGCTCTTCAGCATGAACAGCATCCCTGAGTTCTT-3’ was used to create a mutant with C-terminal sequence GCCS (abbreviated as TR-GCCS) and downstream primer 5’-ACAGCCGCTCTTCAGCATGAACAGCATGACTGAGTTCTT-3’ to create a mutant with C-terminal sequence of SCCS (abbreviated as TR-SCCS).

*Protein production.*

*Escherichia coli* ER2566 cells were used for production of recombinant wild-type (WT) and mutant CeTR2 enzymes. The cells were transformed with plasmid pCETR2, plated on LB-ampicillin plates containing 200 µg/mL ampicillin, and incubated at 37 °C overnight. A single colony isolated from the LB/ampicillin plate was used to grow a 100 mL inoculum culture of LB (200 µg/mL ampicillin). This culture was incubated overnight at 37 °C with shaking. Ten milliliters of inoculum culture was added to each of 6 x 1 L baffled Pyrex Fernbach flasks of TB media containing 200 µg/mL ampicillin. The cells were incubated at 37 °C with shaking (100 rpm) until the OD600 nm reached 1.0. The cells were then chilled on ice until the temperature decreases to 20 °C and induced by adding IPTG to a to final concentration of 0.5 mM followed by incubation at 20 °C
overnight with shaking (100 rpm). The cells were harvested by centrifugation in a Beckman J21B centrifuge (JA-14 rotor) at 7000g at 4 °C for 10 min and then stored at -20 °C.

**Enzyme purification.**

The frozen cell pellets were thawed on ice and resuspended in 200 mL of 50 mM MOPS, 150 mM NaCl, pH 7.0, until homogeneous. The homogenate was sonicated with a Branson 350 Sonifier (Danbury, CT) on ice to break open the cells. The sample was then centrifuged with a Beckman J21B centrifuge (JA-14 rotor) at 7000g for 90 min at 4 °C. The supernatant was gravity loaded onto a 60 mL column of chitin–agarose with a flow rate of 0.5–1.0 mL/min. The column was washed with 50 mM MOPS, 150 mM NaCl, pH 7.0, until the absorbance at 280 nm reached 0.05. This was followed by a 1 L wash of 50 mM MOPS, 500 mM NaCl, pH 7.0. As a final step, the column was then washed with 1 volume of column buffer that contained 140 mM βME (since the fusion protein was resistant to cleavage by βME, I found that a wash with this thiol greatly improved the purity of the protein in this step). The enzyme was then liberated from the intein-fusion protein by incubation of the chitin resin with column buffer (50 mM MOPS, 500 mM NaCl, pH 8.0) containing a small molecule thiol. The thiol-cleavage fractions were concentrated by ultrafiltration using an Amicon Ultra 30000 MWCO (Millipore) concentrator to a final volume of 1–2 mL. The concentrated protein was gravity loaded onto a gel filtration column (S200, Pharmacia) of dimensions 3.8 cm x 97 cm. The gel filtration column was equilibrated with 50 mM MOPS, 500 mM NaCl, pH 7.0. Fractions were collected (3 mL each) and then analyzed by UV–Vis spectrophotometry at 280 and
460 nm. Peak fractions were assayed for DTNB reductase activity and analyzed by SDS–PAGE (12% gels). Peak fractions exhibiting DTNB reductase activity were pooled and concentrated by ultrafiltration. The protein concentration was determined using an extinction coefficient of 22.6 mM⁻¹ cm⁻¹ at 460 nm. The concentrated protein was then stored at 4 °C until needed. The optimal thiol for cleavage was determined by a cleavage assay as described in the next section.

*Optimization of thiol-mediated cleavage.*
Initially, I found the fusion protein to be resistant to cleavage using 70 mM βME as a thiol at pH values ranging from 7 to 8. In order to find the best conditions for thiol mediated cleavage, I explored a variety of thiols and pH values to liberate our target protein (CeTR2) from the chitin–agarose. Four different batches of chitin resin (6 mL slurry) were treated with 15 mL buffer (50 mM MOPS, 500 mM NaCl, pH 7.0) containing either 140 mM βmercaptoethanol (βME), 140 mM N-methylmercaptoacetamide (NMA), 140 mM dithiothreitol (DTT), or 140 mM mercaptoethanesulfonic acid (sodium salt) (MESNA), and incubated at room temperature overnight while mixing using a platform rocker (the pH of the slurry was adjusted to 7.0 after the addition of thiol). A control reaction was done in which buffer was added to the slurry that did not contain thiol. The resin was then filtered and then washed with column buffer that did not contain thiol. The combined effluent was concentrated by ultrafiltration and the samples were then analyzed by 12% SDS–PAGE electrophoresis.

*Enzyme activity assays.*
The activity of the recombinant proteins was tested for their ability to reduce DTNB (5,5’-dithiobis(2-nitrobenzoate)) by monitoring the increase in absorbance at 412 nm (69). The assay contained 4 nM enzyme, 100 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA, 0.2 mM NADPH, and DTNB (0–5 mM) in an assay volume of 0.5 mL. Thioredoxin reductase activity was measured using *E. coli* Trx as substrate, by monitoring the consumption of NADPH by measuring the decrease in absorbance at 340 nm as previously described (70). Briefly, assay mixtures contained 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.15 mM NADPH, 0.16 mM bovine insulin, and *E. coli* Trx (0–420 µM) in a volume of 0.5 mL. The TR concentration was 40 nM for this assay. The data were fit to a straight line over a 2 min range on the Varian-Cary 50 spectrophotometer to calculate the change in absorbance/min.

*Activity towards other substrates.*

WT enzyme was tested for its ability to reduce selenocystine, hydrogen peroxide, and bovine pancreatic insulin. These assays were carried out as previously described with slight modification (70). The assay conditions were: 100 mM potassium phosphate, pH 7.0, 10 mM EDTA, 0.2 mM NADPH, and 200 nM WT enzyme, in a final volume of 0.5 mL for assays using selenocystine or H$_2$O$_2$ as substrates. The assay for bovine pancreatic insulin was tested under the following conditions: 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.15 mM NADPH, and 400 nM WT enzyme in a 0.5 mL assay. Activity was measured by following the decrease in absorbance at 340 nm, indicating consumption of NADPH. The concentration of selenocystine in the assay was 85 µM. Hydrogen
peroxidase activity was measured using concentrations of \( \text{H}_2\text{O}_2 \) varying from 0 to 500 \( \mu \text{M} \). Insulin concentrations were in the range of 0.2–1 mM.

**pH rate profiles.**

WT and mutant enzymes were tested for their ability to reduce *E. coli* Trx at varying pH. The assay was similar to the assay described above with slight modification. In brief, the assay buffer contained 200 mM each of sodium acetate, sodium phosphate (monobasic), sodium phosphate (dibasic), and boric acid. The pH of the buffer was adjusted by 0.5 pH units with HCl or NaOH to give the desired pH value. Insulin was omitted due to precipitation at low pH and each assay reaction contained 500 \( \mu \text{M} \) *E. coli* Trx, 1 mM EDTA, 0.15 mM NADPH, and 60 nM enzyme in a final volume of 0.5 mL.

**Results and Discussion**

*Cloning and Production of Recombinant Enzymes in *E. coli*.*

Sequence analysis of the amplified PCR product indicated that the cloned DNA was identical to that of Genbank accession # Z11115, which was previously identified as the mitochondrial TR from *C. elegans* (62). For production as a recombinant protein in *E. coli*, I removed the mitochondrial signaling sequence (MLLSTFKRHLPIRRL) and fused the enzyme to an intein-chitin binding domain that allows for affinity purification. **Figure 5** details my strategy for isolating the recombinant enzyme. The cell extract was loaded onto a chitin-agarose column and after extensive washing with column buffer, the *C.e. TR2* enzyme was liberated by thiol-mediated cleavage. The liberated protein was eluted from the column with its C-terminus in the form of a thioester. This thioester group hydrolyzes over several days to produce the free carboxylate, typical of C-termini.
of proteins (71). The chitin-agarose column afforded a high level of purification of the enzyme as shown by the 12% SDS-PAGE gel in Figure 6A. The apparent MW of C.e. TR2 is ~55 kDa, typical of high molecular weight thioredoxin reductases (21). The enzyme was then purified to greater than 95% homogeneity (judged by SDS-PAGE) after S200 size-exclusion chromatography (data not shown). The intein-fusion strategy allowed us to purify the enzyme in a two-step process, and also allows us to take advantage of the protein engineering utility of inteins for future experiments (72). The intein strategy has proven useful to us in the past, however in the case of C.e. TR2, this strategy proved to be challenging as cleavage from the intein through the use of a thiol proved difficult. Figure 6B shows the cleavage of mouse TR2 from an intein fusion protein using different thiols. The thiols βME, NMA, and DTT all cleaved with similar efficiency, while MESNA cleaved with the least efficiency. Initially I tried to cleave the C.e. TR2-intein fusion protein with βME, which resulted in little or no cleavage. The results of our thiol cleavage assay in Figure 6A demonstrate the superiority of NMA as a cleavage thiol. DTT and βME did not cleave the construct to any significant extent at either pH 7.0 or pH 8.0. MESNA (data not shown) also demonstrated an inability to cleave the fusion protein. However, as can be seen in Figure 6A, NMA cleaved the fusion protein to a significant extent at pH 7.0 and allowed us to use the intein strategy as a method for isolating the recombinant enzyme with a final yield of 2 mg/L of cell culture. The purified WT and mutant enzymes absorbance spectra were scanned from 200 to 600 nm (data not shown), with characteristic maxima at 370 nm and 460 nm for the flavin-bound cofactor present.
**Thioredoxin Reductase Activity.**

The thioredoxin reductase activity of *C.e.* TR2 was determined using *E. coli* Trx as substrate. *E. coli* Trx has been used as a standard to measure the thioredoxin reductase activity of many different types of TR’s, however it has been shown that TR’s from different species prefer their cognate thioredoxin as a substrate, and the preference for the cognate thioredoxin molecule can vary greatly between species (25).

This activity was measured using a well-developed assay as described by Holmgren (70). The results of the assay containing NADPH, bovine insulin, and *E. coli* Trx in 50 mM potassium phosphate buffer for WT and mutant *C.e.* TR’s are shown in **Figure 7** as a Michaelis-Menten plot and the kinetic data is summarized in **Table 1**. The results show that the WT enzyme has high activity towards *E. coli* Trx as measured by its high value of $k_{\text{cat}}$ ($610 \text{ min}^{-1}$). However, the WT enzyme also has a high $K_m$ value (610 µM) towards *E. coli* Trx, indicating that the non-cognate Trx is not the optimal substrate for the enzyme. It is likely that the *C.e.* TR2 would bind its cognate Trx with much higher affinity resulting in a lower $K_m$ value. This result is similar to the results of the TR from *Plasmodium falciparum* (*P.f.*), which also has high activity towards *E. coli* Trx, but also displays a high $K_m$ value (67). When the *P.f.* TR is assayed using its own cognate Trx, the $k_{\text{cat}}$ value is similar to the $k_{\text{cat}}$ value when *E. coli* Trx is the substrate, but the $K_m$ value for the cognate Trx is much lower (**Table 2**). The high value of $k_{\text{cat}}$ towards *E. coli* Trx does indicate that like the TR’s from *Drosophila melanogaster* and *Plasmodium falciparum*, the reduction of the catalytic disulfide bond of Trx does not require selenium, and furthermore that the enzymes containing an active-site thiolate nucleophile are
capable of achieving catalytic rates comparable to the mammalian enzymes which contain Sec.

The WT enzyme has unusual kinetic properties at low substrate concentration (0 – 30 µM). When measuring the absorbance at 340 nm during the assay, the absorbance should decrease indicating consumption of NADPH and turnover of substrate. However at low substrate concentration, the absorbance was observed to increase. This either indicates that the enzyme has NADPH oxidase activity at low substrate concentration, or the enzyme exhibits cooperativity. Alternatively, this result may be an artifact of using a non-cognate Trx as substrate. Neither mutant enzyme displayed this property.

The mitochondrial TR from *C. elegans* (*C.e.* TR2) is of interest because of the proposal by Arnér and coworkers that *D.m.* TR can achieve high catalytic activity without the need for Sec because of the presence of flanking serine residues in the C-terminal tetrapeptide SCCS redox center of the enzyme(60). These serine residues take the place of flanking glycine residues in the mammalian C-terminal tetrapeptide motif of GCUG. It was proposed that the serine residues act as general bases with the assistance of a nearby histidine residue to deprotonate the Cys residues during the catalytic cycle of the enzyme. *C.e.* TR2 is a naturally occurring Cys-containing TR (Cys replaces Sec in the active site) that has a flanking glycine residue on each side of the vicinal Cys motif. Thus in the case of *C.e.* TR2, there is no requirement for flanking serine residues to assist in proton abstraction and the enzyme can achieve high activity in the absence of Sec. The mechanism of *D.m.* TR may require the presence of flanking serine residues to achieve catalysis, but this is certainly not the case for the *C.e.* TR2.
I have also constructed several mutants to further explore the hypothesis put forth by Arnér and coworkers that flanking serine residues are essential for TR’s with vicinal Cys residues\(^\text{60}\). In *D.m.* TR, it was shown that the C-terminal serine was more important to catalysis since mutation of this residue to glycine affected the catalytic activity the most. When I changed the C-terminal glycine residue of *C.e.* TR2 to serine I did not observe an increase in activity as might be expected if the presence of a serine in this motif could function to deprotonate the C-terminal Cys residue as in the Arnér proposal. In fact, the activity of the enzyme decreased 8-fold in *k*\(_{\text{cat}}\), however, this mutant did show better binding of the substrate as *K*\(_{m}\) decreased nearly 2-fold. Thus this residue may be important for substrate binding and further differences in *K*\(_{m}\) may be observed with this mutant when comparing *E. coli* Trx to the cognate Trx from *C. elegans*. However, the mitochondrial Trx from *C. elegans* has not been cloned and as far as I know is unavailable. This is an area for future work. When both flanking glycine residues are mutated to serine (the opposite experiment to that in ref. \((60)\)), the activity is 3 fold lower in *k*\(_{\text{cat}}\) and has a 2.5-fold lower *K*\(_{m}\). The results show that similar to the proposal of Arnér and coworkers, the C-terminal flanking residue seems to be more important for catalysis than the N-terminal flanking residue in *C.e.* TR2.

*DTNB reductase activity.*

DTNB has been widely used as a substrate for thioredoxin reductases as the disulfide bond of this substrate is highly reactive and this provides a convenient method for assaying the enzyme during purification. The catalytic constants using DTNB are usually reported as a means of comparing the activity of different TR’s towards a
The Michaelis-Menten plot for *C.e.* TR2 using DTNB as a substrate is shown in **Figure 8**. The enzyme has a $k_{cat}$ of 134 min$^{-1}$ and a $K_m$ of 0.42 mM. This compares to a $k_{cat}$ of 2,100 min$^{-1}$ and a $K_m$ of 100 mM for the mammalian Sec-containing enzyme (73). The $k_{cat}$ of the *C.e.* TR2 for DTNB is similar to that of the mammalian U498C mutant (Sec to Cys mutation) (220 s$^{-1}$) (73). Sometimes a specific activity for DTNB at a concentration of 5 mM is reported in the literature (59). For example, calf liver TR has a specific activity of 1,333 mol NADPH/min/mol TR at 5 mM DTNB, while the corresponding value for *C.e.* TR2 is 130. This most likely reflects the poorer nucleophilicity of the active site thiolate of *C.e.* TR2 compared to the selenolate for the mammalian enzyme.

*C.e.* TR2 was also tested for its ability to reduce several other substrates (**Table 3**). The enzyme showed no detectable hydrogen peroxidase activity and no ability to reduce disulfide bonds of peptides such as in insulin. The lack of hydrogen peroxidase activity is consistent with previous reports of the requirement for selenium to catalyze this reaction and reflects the poorer nucleophilicity towards peroxide bonds by sulfur in comparison to selenium (74). The enzyme was able to utilize selenocystine as a substrate and I report a specific activity of 29 min$^{-1}$ using 85 µM selenocystine.

**pH Rate Profile.**

WT and mutant enzymes were tested for their ability to reduce *E. coli* Trx at varying pH. The WT enzyme was found to have a pH optimum centered near pH 8.0 (**Figure 9**). Mutant enzymes containing altered C-terminal tetrapeptides (GCCS and SCCS) also showed a pH optimum near 8.0, but the mutant profiles are somewhat
broader than the WT enzyme. The mammalian Sec-containing enzyme shows a pH optimum near 7.0, while the pH optimum is shifted between 8-10 for the mammalian U498C mutant (59). The C.e. TR2 enzyme, like the mammalian U498C mutant also shows a pH optimum shifted towards 8.0, but the profile is narrower compared to the Cys-containing mammalian mutant. C.e. TR2 likely evolved to utilize the nucleophilicity of an active-site thiolate, potentially through the use of a general base.

The mitochondrial TR of the nematode C. elegans has evolved down a different path than that of the mammalian mitochondrial enzyme. While the cytosolic C. elegans TR1 contains Sec, its mitochondrial counterpart does not. It is not known why C. elegans has evolved to contain the entire Sec decoding apparatus for one Sec-containing enzyme (75). One possibility for the mitochondrial C.e. TR is that it originally contained Sec, but was lost through evolution and was replaced with Cys. It has been proposed that the occurrence of Sec in proteins is the result of a recent evolutionary event (76) and that the presence of Sec in TR enables the enzyme to have additional biological roles inside the cell other than the reduction of Trx (77). Thus, the function of the mitochondrial TR from C. elegans may be limited to the reducing the catalytic disulfide bond of Trx because the redox-sensing function of TR may be performed by other proteins in this organelle of C. elegans.

Conclusions
In conclusion, the C.e. TR2 enzyme containing Cys in place of Sec has high catalytic activity towards E. coli Trx, as has been observed for the Cys-containing TR of D. melanogaster (25). The C.e. TR2 enzyme must use a mechanism that is slightly
different from that of the *D.m.* TR since no requirement for flanking serine residues is observed.
Table 1: Kinetic Constants for WT and Mutant Enzymes using *E. coli* Trx as Substrate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat} / K_m$ (min$^{-1}$•M$^{-1}$)</th>
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<tr>
<td><em>C. e.</em> TR-GCCG (WT)</td>
<td>610 ± 36</td>
<td>610 ± 60</td>
<td>1.0 x10$^5$</td>
</tr>
<tr>
<td><em>C. e.</em> TR-GCCS</td>
<td>318 ± 25</td>
<td>77 ± 3</td>
<td>2.4 x10$^5$</td>
</tr>
<tr>
<td><em>C. e.</em> TR-SCCS</td>
<td>242 ± 33</td>
<td>219 ± 14</td>
<td>9.0 x10$^5$</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Substrate</td>
<td>$K_m$ (µM)</td>
<td>$k_{cat}$ (min$^{-1}$)</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>rTR-GCCUG$^a$</td>
<td>$H. sapiens$ Trx</td>
<td>3.3</td>
<td>2500</td>
</tr>
<tr>
<td>rTR-GCCG$^a$</td>
<td>$H. sapiens$ Trx</td>
<td>0.4</td>
<td>14.3</td>
</tr>
<tr>
<td>C.e. TR-GCCG</td>
<td>$E. coli$ Trx</td>
<td>610</td>
<td>610</td>
</tr>
<tr>
<td>P.f. TR-GCGGGKCG$^b$</td>
<td>P.f. Trx</td>
<td>2.1</td>
<td>1674</td>
</tr>
<tr>
<td>P.f. TR-GCGGGKCG$^b$</td>
<td>$E. coli$ Trx</td>
<td>500</td>
<td>1688</td>
</tr>
<tr>
<td>D. m. TR-SCCS$^c$</td>
<td>$D. m.$ Trx</td>
<td>7.1</td>
<td>1320</td>
</tr>
<tr>
<td>A.g TR-TCCS$^d$</td>
<td>A.g. Trx</td>
<td>8.5</td>
<td>924</td>
</tr>
</tbody>
</table>

$^a$Taken from ref. (59).
$^b$Taken from ref. (67).
$^c$Taken from ref. (25).
$^d$Taken from ref. (61).
### Table 3: Activity of C. elegans TR2 vs. Various Substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (mol NADPH/min/mol of TR)</th>
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<tr>
<td>DTNB</td>
<td>130</td>
</tr>
<tr>
<td>Selenocystine (85 µM)</td>
<td>29</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>No activity</td>
</tr>
<tr>
<td>Insulin</td>
<td>No activity</td>
</tr>
</tbody>
</table>
Figure 5: Fusion protein mechanism for affinity purification
The target protein, *C.e. TR*, was fused at its carboxy terminus with an engineered intein. The construct also has a chitin-binding domain (CBD) at the C-terminus, which allows for affinity purification with chitin-agarose. The CBD is a small peptide that has high affinity for chitin-agarose, and this resin provides a high level of purification of the fusion protein. The Cys residue (of the intein) at the TR-intein junction, undergoes an N-S acyl shift resulting in the formation of a thioester bond at the TR-intein junction. Addition of a small thiol (NMA) mediates cleavage of TR from the intein, which remains bound to the chitin resin through the CBD. The liberated thioester tagged protein then undergoes hydrolysis yielding a native carboxy terminus.
Figure 6: Thiol mediated cleavage efficiency of different small molecule thiols.
In (A), C.e. TR2 is cleaved with 140 mM thiol at pH 7.0. Lane 1: MWM. Lane 2: Fresh chitin resin without protein that has been treated with a 1% SDS solution as a control. Lane 3: The column wash (buffer) after fresh chitin resin has been loaded with protein extract. Lane 4: 1% SDS wash from a chitin column loaded with protein extract. Lane 5: βME wash of chitin column. Lane 6: 1% SDS wash of column following βME wash. Lane 7: DTT wash of chitin column. Lane 8: 1% SDS wash of column following DTT wash. Lane 9: NMA wash of chitin column. Lane 10: 1% SDS wash of column following NMA wash. The liberated C.e. TR2 is indicated by the arrow showing the band with an apparent MW of 55 kDa. In (B), mouse TR3 is produced as an intein fusion and cleaved with different thiols (50 mM) at pH 7.0. Lane 1: βME. Lane 2: MWM. Lane 3: NMA. Lane 4: DTT. Lane 5: MESNA. In (B) NMA, DTT, and βME all cleave with comparable efficiencies, with MESNA being the least efficient cleavage thiol. In (A) the superiority of NMA as a cleavage thiol is evident. In (C), the structures of each thiol (from top to bottom: βME, NMA, DTT, and MESNA), along with their corresponding $pK_a$ values are shown (78).
Figure 7: Michaelis-Menten plot of WT and mutant enzymes using thioredoxin as substrate.

Purified WT and mutant *C. elegans* TR’s were assayed for their ability to reduce *E. coli* Trx. The activity was monitored by consumption of NADPH via a decrease in absorbance at 340 nm. Reaction mixtures contained 50 mM potassium phosphate pH 7.0, 1 mM EDTA, 150 µM NADPH, 160 µM bovine pancreatic insulin, 1-450 µM *E. coli* Trx and 40 nM enzyme. The WT enzyme is represented by the closed circles (TR-GCCG), open squares represent TR-GCCS, and closed diamonds represent TR-SCCS.
Figure 8: Michaelis-Menten plot of WT CeTR2 using DTNB as substrate.
The assay contained 100 mM potassium phosphate, pH 7.0, 10 mM EDTA, 200 µM NADPH, 4 nM enzyme, and 0-5.0 mM DTNB. Reactions were monitored over 2 min for an increase in absorbance at 412 nm indicating reduction of DTNB. Activity is expressed as mol of NADPH consumed/min/mol TR.
Figure 9: Activities of WT and mutant CeTR2 as a function of pH.
The thioredoxin reductase activity of WT and mutant enzymes was measured in buffers of varying pH. The activity was measured in 0.5 pH unit increments and is expressed as a percent of the maximal activity. Reaction mixtures contained 200 mM each of potassium phosphate (monobasic), potassium phosphate (dibasic), sodium acetate, and boric acid along with 1 mM EDTA, 150 µM NADPH, 60 nM enzyme, and 500 µM E. coli Trx. The WT enzyme is represented by the closed circles (TR-GCCG), open squares represent TR-GCCS, and closed diamonds represent TR-SCCS.
Chapter 3  
C-Terminal Mutagenesis Studies of *C. elegans* Mitochondrial Thioredoxin Reductase

**Introduction**

As discussed in Chapter 1, the C-terminal vicinal Cys residues form a strained eight-membered ring. Since there are several examples of *M. trichosporum* TR that have similar functional catalytic activities, compared to the mammalian Sec containing TRs, I want to evaluate the role of this ring in catalysis.

Since the eight-membered ring motif is rare in nature, I wanted to test the effect of changing the conformation of the ring by making it larger. The use of PCR mutagenesis was performed to insert either one or two alanine residues between the vicinal Cys residues. This resulted in eleven and fourteen-membered rings (Error! Reference source not found.). These constructs were assayed for DTNB, and Trx reductase activity. Additionally, I examined their pH dependence on the reduction of *E.coli* Trx.

**Methods**

*Cloning of His-Tagged CeTR2.*

Plasmid pCeTR2 (26) was used as a template to clone the CeTR2 gene into plasmid pET45b(+) (Novagen) using an upstream primer containing a “hexaHis-tag”. For this construct, an upstream primer of sequence 5′-ACAGCCCCGGATCCCTTCTCATC AAATAAATTTGATCTG-3′ was used with downstream primer, 5′-ACAGCCAAGCTTGTCGACTCATCCACACGCTCCCTGAGTTTCTTGG-3′ in the
amplification process to produce the WT enzyme with C-terminal sequence Gly-Cys-Cys-Gly. C-terminal mutants were produced using downstream primers 5′-ACAGCCAAGCTTGTGACTCATCCACATGCGCATCCCTGAGTTCTTGG-3′ (Gly-Cys-Ala-Cys-Gly mutant), and 5′-ACAGCCAAGCTTGTGACTCATCCACATGCGCATCCCTGAGTTCTTGG-3′ (Gly-Cys-Ala-Ala-Cys-Gly mutant). The resulting plasmids (pHisCeTR2) encode a protein with a N-terminal hexa-histidine tag followed by an enterokinase cleavage site and the CeTR2 protein.

The PCR reaction mixtures contained 100 pg template DNA, 50 pmol of each primer, 2 units of Vent DNA Polymerase, 2 mM magnesium chloride, in a volume of 100 µL. Each PCR was performed on a GeneAmp PCR System 2400 from Perkin Elmer Life Sciences, Inc. (Boston, MA) using 25 cycles with the following parameters: 96 °C for 45 sec, 50 °C for 30 sec, and 72 °C for 180 sec. The product was analyzed by analytical agarose gel electrophoresis and then purified using the QIAquick Purification Kit from Qiagen (Valencia, CA).

The PCR product and pET45b(+) plasmid were then treated with restriction enzymes Bam HI and Sal I for 2 h at 37°C. Digests were purified using the Qiagen QIAquick PCR purification kit. Ligation reactions contained 200 ng of plasmid and 400 ng of PCR product and were incubated at 16 °C for 16 h using T4 DNA ligase. Following heat inactivation of T4 DNA ligase for 15 min at 70 °C, the ligation reaction was then incubated at 37 °C with 5U Asc I for 2 h to enhance the amount of positive clones as Asc I will only cut plasmids that have not been ligated with the PCR insert. E. coli DH5α
cells were made competent via the Inoue method (79) and then transformed with 1 µL of purified, background digested, DNA. DNA from isolated colonies were purified using QIAprep Spin Minprep Kit (Qiagen) and screened by restriction analysis using Bam HI and Sal I, analyzed by analytical 0.8% agarose gel electrophoresis, and positive clones were verified by sequencing of the DNA coding region at the DNA Analysis Core Facility, Vermont Cancer Center, University of Vermont.

**Purification of WT and Mutant CeTR2 Enzymes.**

For expression of recombinant WT CeTR2 and mutant enzymes, *E. coli* ER2566 cells were used. The cells were transformed with the plasmid, plated on LB-ampicillin plates containing 200 µg/mL ampicillin, and incubated at 37 °C overnight. A single colony was used to grow a 100 mL inoculum culture of LB (200 µg/mL ampicillin). This culture was incubated overnight at 37 °C with shaking. An inoculum culture of 10 mL was added to a 1 L baffled Pyrex Fernbach flask containing TB media (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 16 mM potassium phosphate monobasic and 72 mM potassium phosphate dibasic at pH 7.0 and supplemented with 20 mg/L each of niacinamide, pyridoxine, and riboflavin) containing 200 µg/mL ampicillin. The cells were incubated at 37 °C with shaking (100 rpm) until the O.D. at 600 nm reached 1.0. The cells were then chilled on ice until the temperature decreased to 20 °C and then induced by adding IPTG to a to final concentration of 0.5 mM. The induced cells were incubated at 20 °C overnight with shaking (100 rpm). The cells were harvested by centrifugation and stored at –20 °C.
The frozen cell pellets were thawed on ice and homogenized in 100 mL of 50 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 20 mM βME, pH 8.0, until homogeneous. Lysozyme was added to the homogenate at a concentration of 1 mg/mL and allowed to stir at 4 °C for 1 h. The homogenate was then briefly sonicated (5 x 30 sec). The sample was then centrifuged with a Beckman J21B centrifuge (JA-14 rotor) at 7000 x g for 90 min at 4 °C. The supernatant was gravity loaded onto a 5 mL column of Ni-NTA resin with a flow rate of 0.5–1.0 mL/min. The column was washed with the buffer until the absorbance at 280 nm reached 0.05. This was followed by a wash with buffer containing 20 mM imidazole to eliminate nonspecific protein binding after which the protein was eluted in a buffer containing 250 mM imidazole.

The protein was dialyzed against modified TE buffer (10 mM Tris, 1 mM EDTA, 10 mM NaCl, pH 8.0, 2 x 4L). The dialyzed protein was then reduced by addition of 20 mM βME and then loaded on to a 2',5'-ADP Sepharose column (20 mL, Amersham) equilibrated with 10 mM Tris, 1 mM EDTA, 10 mM NaCl, 20 mM βME, pH 8.0 and washed until the A_{280} was below 0.05. Protein was eluted in buffer containing 3 M NaCl and analyzed by 12% SDS-PAGE. Fractions were pooled and concentrated by ultrafiltration using an Amicon Ultra 30,000 molecular weight cut off (MWCO) (Millipore) concentrator to a final volume of 1–2 mL. The concentrated protein was gravity loaded onto a Sephacryl S-200 gel filtration column (Pharmacia) of dimensions 3.8 cm x 97 cm. The column was equilibrated with 50 mM potassium phosphate, 300 mM NaCl, and 1 mM EDTA at pH 8.0. Fractions were collected (4 mL each) and then analyzed by UV–Vis spectrophotometry at 280 and 460 nm and examined for purity by
12 % SDS–PAGE. Fractions exhibiting the 55 kD band were pooled and concentrated by ultrafiltration.

Production of Thioredoxin.

The clone containing the gene for TrxA from *E. coli* was a gift from Ronald T. Raines (80) and was produced as previously described (81). *E. coli* BL21(DE3) cells made competent with CaCl₂ and transformed with 50 ng of DNA and plated onto LB agar supplemented with 200 μg/mL ampicillin. Single colonies were used to inoculate 100 mL ampicillin-containing LB media and allowed to shake overnight at 37 °C.

Six liters of LB media (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with 200 μg/mL ampicillin was inoculated with 10 mL (each liter) of inoculum culture and grown to an O.D. of 0.6 at 600 nm while shaking at 37 °C in a C25KC shaker incubator (New Brunswick Scientific). The cells were induced for 3 h at 37 °C with 0.5 mM IPTG, and then harvested by a 10 min centrifugation at 10,000 rpm in a JA-14 rotor using a Model J-21B centrifuge from Beckman. The resulting cell pellets were frozen at –20 °C. The cells were then thawed on ice, homogenized in 20 mM Tris, pH 8.4 containing 1 mM EDTA, and lysed by probe sonication using a Branson Sonifier. The lysate was centrifuged as described above for 90 min and the resulting supernatant was loaded onto a 70 mL column of DEAE sephacel (Sigma-Aldrich) equilibrated with the lysis buffer. After loading, the column was washed with buffer until the A₂₅₀ of the effluent was 0.05. The sample was eluted using two 400 mL gradients containing NaCl (0 mM to 100 mM and 100 mM to 250 mM). Fractions were collected (3 mL) and evaluated by absorbance at 280 nm and further analyzed by 15% SDS-PAGE. Fractions containing
Trx were pooled and then adjusted to 60% ammonium sulfate saturation and centrifuged as above for 60 min. The pellet was solubilized in a minimal volume of 20 mM Tris pH 8.4, 250 mM NaCl, 1 mM EDTA.

A 2 mL sample was loaded onto a Sephacryl S-100 HR, 98 cm x 3.3 cm, gel filtration column from Pharmacia-Amersham Biosciences (Uppsala, Sweden) equilibrated with sample buffer. Collected fractions (1 mL) were evaluated by absorbance at 280 nm and SDS-PAGE. The fractions containing Trx were pooled, concentrated using an Amicon Ultra with 5000 MWCO from Millipore. Purity was > 95% as judged by 15% SDS-PAGE. The Trx concentration was calculated using the extinction coefficient at 280 nm of 13,700 M⁻¹cm⁻¹ (82).

*Enzymatic Characterization of Thioredoxin Reductase.*

The TR mutants were assayed for activity towards DTNB, and Trx, as described by Arner (70). All assays were performed on a Cary 50 UV/VIS spectrophotometer from Varian (Walnut Creek, CA) at 25 °C, pH 7.0, and were initiated by addition of enzyme. Spectral properties of purified TR were evaluated at 275 nm, 370 nm, 460 nm and the concentration of homodimeric TR was determined using the flavin extinction coefficient at 460 nm of 22.6 mM⁻¹cm⁻¹ (70). Activity was monitored over 2 min with Vₒ determined from the linear fit. Plots of Vₒ/Eₚ vs. substrate concentration were fit by the Michaelis-Menten equation using KaleidaGraph 4.02 from Synergy Software (Reading, PA) and activities reported as moles of NADPH consumed per min per mole of TR dimer.

The DTNB assay contained 0.2 mM NADPH and 10 mM EDTA in 100 mM potassium phosphate. For each concentration of DTNB, activity was corrected for
background by addition of buffer only. Activity was measured by the increase in absorbance at 412 nm, calculated using the extinction coefficient for TNB-S' (5-thio-2-nitrobenzoate, 13.6 mM$^{-1}$•cm$^{-1}$), and divided by two to account for the production of 2 TNB-S$^-$ per NADPH consumed. The concentration of CeTR2 was 4 nM.

The Trx assay contained 0.15 mM NADPH, 1 mM EDTA, and 10 mg/mL insulin in 50 mM potassium phosphate in a volume of 500 µL. Activity was background corrected for each concentration of Trx by addition of buffer only as well as with enzyme in the absence of substrate. Activity was measured by the decrease in absorbance at 340 nm for the consumption of NADPH and calculated using the extinction coefficient of 6200 M$^{-1}$•cm$^{-1}$. The concentration of WT CeTR2 in the assay was 40 nM. The concentration of each mutant TR was adjusted to achieve a similar change in absorbance at 340 nm as that of their respective WT TR. The single and double alanine insertion mutants displayed poor Trx activity and required 1600nM and 800nM of homodimeric TR in the assay, respectively.

**pH Optima of Trx Reduction.**

Activity towards Trx was tested as a function of pH for each construct. Due to the insolubility of insulin below pH 7.0, each assay utilized 500 µM Trx, which is approximately ten times greater than the estimated $K_m$. To avoid differences in ionic strength, each assay contained buffer with a final concentration of 30 mM citrate/30 mM Tris/30 mM phosphate adjusted from pH 5.5 to 10.0. The concentration of TR in the assay was the same as used for the Trx Michaelis-Menten profile. Each assay contained 0.15 mM NADPH and 1 mM EDTA. The activity was measured at 340nm and
The data were collected in duplicate and then normalized to the percent of maximal activity for each given mutant and plotted as percent activity vs. pH.

**Results and Discussion**

*Thioredoxin Reductase Activity for CeTR.*

Increasing ring size of the Cys-Cys dyad by inserting alanine residues resulted in a 145 fold decrease in \( k_{\text{cat}} \) for the single insertion and 90 fold decrease for the double insertion respectively, while the \( K_m \) was little affected compared to WT CeTR2 ([Table 4, Figure 11](#)). Similar results are observed for DmTR, which also had a greatly reduced activity 150 to 300 fold loss in \( k_{\text{cat}} \), compared to WT DmTR ([Table 5](#)). Similar results were not observed with mTR3, where increasing the ring size of the Cys-Sec dyad by inserting alanine residues had only a modest effect on activity (4-6 fold lower) ([Table 6](#)). These results indicate that the non-selenium enzymes have a greater dependence on a vicinal disulfide than does the mammalian enzyme for a vicinal selenylsulfide.

*Comparison of DTNB Reductase Activities.*

The small molecule disulfide DTNB has long been used for quantification of free thiols in proteins (83) as well as to evaluate thiol-disulfide exchange reactions (84), as is the process catalyzed by TR. As a substrate of TR, DTNB displays Michaelis-Menten kinetics, but it is not a physiologically relevant substrate, which is reflected by the high \( K_m \) values that have been previously reported for this substrate (59, 85, 86). It does, however, provide a substrate suitable for evaluating the activity of TR mutants that show little or no activity towards Trx since both the N-terminal and C-terminal disulfide redox centers are capable of reducing DTNB as has been reported by us (72), and others (85, 48).
A good model for either redox center interacting with DTNB has been previously presented (73).

For our truncated form of CeTR2 (Table 7, Figure 12), I observe a $k_{\text{cat}}$ of $152 \pm 14$ min$^{-1}$. This is 113% of the WT enzyme, which has a $k_{\text{cat}}$ of $134 \pm 7$ min$^{-1}$. The WT and truncated CeTR2 have comparable $K_m$ values. Like the truncated CeTR2, the alanine insertion mutants also display high activity compared to the WT enzyme. For the single and double insertion mutants I observe a $k_{\text{cat}}$ of $107 \pm 4$ min$^{-1}$ and $223 \pm 14$ min$^{-1}$, which result in 70% and 147% of the WT CeTR2 enzyme. This same effect is observed in DmTR, where alanine insertion between the redox-active Cys residues of the C-terminal redox center has very little effect on DTNB reductase activity (Error! Reference source not found.). The Cys-containing DmTR and CeTR2 enzymes have $\sim$12% of the DTNB reductase activity of the Sec-containing mTR3 ($k_{\text{cat}}$ of $157$ min$^{-1}$ for DmTR and $1251 \pm 71$ min$^{-1}$ for mTR3, respectively), while their catalytic efficiencies are only 3-fold and 8-fold lower than mTR3, respectively.

For the truncated form of mTR3 (Table 9), I observe a $k_{\text{cat}}$ of $856 \pm 43$ min$^{-1}$. This is 68% of the semisynthetic WT enzyme, which has a $k_{\text{cat}}$ of $1251 \pm 71$ min$^{-1}$. The WT does however, have a significantly lower $K_m$. This is different from what is observed for the C-terminal mutant of rat TR1 (59) or the truncated form of human TR (87), which show poor activity. However, this result is similar to a C-terminal mutant of Plasmodium falciparum TR (PfTR), which had 64% of WT DTNB reductase activity (86). The alanine insertion mutants (TR-GCAUG and TR-GCAAUG) have slightly elevated activities compared to the truncated enzyme, but less than the semisynthetic WT enzyme.
**pH Data**

The effect of enlarging the C-terminal ring had on the pH rate profile for the reduction of *E.coli* Trx was examined. WT CeTR2 data indicates a pH optimum of 7.5, while for both alanine insertion mutants there was distinct shift in the optimum toward the alkaline centered between pH 8.0-8.5 (Figure 13).

**Conclusions**

This investigation examined TRs C-terminal eight-membered ring motif and what affect enlarging the ring would have on the reduction *E.coli* Trx. From the data, I conclude that for CeTR2, the size of the ring is an important factor for the reduction of *E.coli* Trx. Another member of the Cys-containing TR, DmTR, similar results was found. When the ring of the Sec-containing TR, mTR3, was enlarged similarly to the Cys-containing TRs, activity was not dramatically affected. I conclude that the eight-membered ring structure is essential for the Cys-containing TRs, to have high Trx reduction activity, while for mTR3 it is not.
Table 4: CeTR2 thioredoxin reductase activity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{a}$CeTR2-GCCG-cOO- (Intein)</td>
<td>610 ± 36</td>
<td>610 ± 60</td>
</tr>
<tr>
<td>CeTR2-GCCG-cOO-</td>
<td>379 ± 21</td>
<td>247 ± 26</td>
</tr>
<tr>
<td>CeTR2-GCAG-cOO-</td>
<td>2.6 ± 0.14</td>
<td>111 ± 17</td>
</tr>
<tr>
<td>CeTR2-GCAAAG-cOO-</td>
<td>4.2 ± 0.35</td>
<td>142 ± 31</td>
</tr>
<tr>
<td>$^b$CeTR2-Truncated</td>
<td>No Activity</td>
<td>No Activity</td>
</tr>
</tbody>
</table>

$^{a}$I previously reported the activity of CeTR2 produced as an intein-fusion protein in (26). In the present study, I have used a hexaHis-tag for affinity purification instead of the intein and I note that the hexaHis-tag enzyme has lower activity.

$^{b}$Truncated CeTR2 lacking C-terminal eight amino acids that compose the eight-membered ring motif.
Table 5: DmTR thioredoxin reductase activity.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^b$TR-S-COO-</td>
<td>No activity</td>
<td>No activity</td>
</tr>
<tr>
<td>$^c$TR-SCCS-COO-</td>
<td>299.4 ± 7.4</td>
<td>173.3 ± 8.1</td>
</tr>
<tr>
<td>TR-SCACS-COO-</td>
<td>2.12 ± 0.3</td>
<td>298.3 ± 58.0</td>
</tr>
<tr>
<td>TR-SCAACS-COO-</td>
<td>0.91 ± 0.2</td>
<td>166 ± 58.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data taken from Ref. (88)

\textsuperscript{b}The truncated form of the enzyme missing the C-terminal tripeptide Cys-Cys-Ser.

\textsuperscript{c}The WT enzyme.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>% Peptide Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^b,c$ mTR3-G-COO-</td>
<td>No activity</td>
<td>No activity</td>
<td>NA</td>
</tr>
<tr>
<td>$^b,d$ mTR3-GCG-COO-</td>
<td>4.1 ± 0.11</td>
<td>49.1 ± 3.2</td>
<td>NA</td>
</tr>
<tr>
<td>$^b,e$ mTR3-GCUG-COO-</td>
<td>2220 ± 78</td>
<td>67.6 ± 6</td>
<td>91</td>
</tr>
<tr>
<td>mTR3-GCAUG-COO-</td>
<td>350 ± 14</td>
<td>20.8 ± 3.5</td>
<td>100</td>
</tr>
<tr>
<td>mTR3-GCAAUG-COO-</td>
<td>501 ± 41</td>
<td>34.9 ± 10.0</td>
<td>100</td>
</tr>
</tbody>
</table>

$^a$Date taken from Ref. (88)
$^b$Reported previously in (72).
$^c$The truncated form of the enzyme missing the C-terminal tripeptide Cys-Sec-Gly.
$^d$The full length mutant Sec489Cys.
$^e$The WT enzyme produced by semisynthesis.
Table 7: DTNB reductase activity for CeTR2.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_{m}$ (mM)</th>
<th>$k_{\text{cat}} / K_{m}$ (min$^{-1}$•mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{a}$CeTR2-<strong>GCCG-coo-</strong> (Intein)</td>
<td>134 ± 5.2</td>
<td>0.42 ± 0.07</td>
<td>319</td>
</tr>
<tr>
<td>CeTR2-<strong>GCCG-coo-</strong></td>
<td>134 ± 7.1</td>
<td>0.41 ± 0.09</td>
<td>327</td>
</tr>
<tr>
<td>CeTR2-<strong>CACG-coo-</strong></td>
<td>107 ± 3.6</td>
<td>0.22 ± 0.04</td>
<td>486</td>
</tr>
<tr>
<td>CeTR2-<strong>CAACG-coo-</strong></td>
<td>223 ± 13.8</td>
<td>0.37 ± 0.10</td>
<td>603</td>
</tr>
<tr>
<td>CeTR2-Truncated-coo-</td>
<td>152 ± 14</td>
<td>0.53 ± 0.19</td>
<td>287</td>
</tr>
</tbody>
</table>

$^a$Previously reported in (26).

$^b$Truncated CeTR2 lacking C-terminal eight amino acids that compose the eight-membered ring motif.
Table 8: DTNB reductase activity for DmTR.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}} / K_m$ (min$^{-1}$•mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DmTR-S-COO-</td>
<td>178.0 ± 7.0</td>
<td>0.75 ± 0.09</td>
<td>237</td>
</tr>
<tr>
<td>DmTR-SCCS-COO-</td>
<td>157 ± 12.4</td>
<td>0.22 ± 0.07</td>
<td>713</td>
</tr>
<tr>
<td>DmTR-SCACS-COO-</td>
<td>187.5 ± 17</td>
<td>0.20 ± 0.06</td>
<td>935</td>
</tr>
<tr>
<td>DmTR-SCAAAC-S-COO-</td>
<td>94.9 ± 4.1</td>
<td>0.25 ± 0.03</td>
<td>380</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data taken from Ref. (88)
\textsuperscript{b}The truncated form of the enzyme missing the C-terminal tripeptide Cys-Cys-Ser.
\textsuperscript{c}The WT enzyme.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_{\text{m}}$ (mM)</th>
<th>$k_{\text{cat}} / K_{\text{m}}$ (min$^{-1}$•mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^a$mTR3-G-COO-</td>
<td>856 ± 43</td>
<td>2.72 ± 0.43</td>
<td>314</td>
</tr>
<tr>
<td>$^c$mTR3-GCG-COO-</td>
<td>794 ± 78</td>
<td>1.75 ± 0.41</td>
<td>454</td>
</tr>
<tr>
<td>$^d$mTR3-GCUG-COO-</td>
<td>1251 ± 71</td>
<td>0.46 ± 0.09</td>
<td>2720</td>
</tr>
<tr>
<td>mTR3-GCAUG-COO-</td>
<td>1010 ± 26</td>
<td>0.26 ± 0.03</td>
<td>3885</td>
</tr>
<tr>
<td>mTR3-GCAAUG-COO-</td>
<td>999 ± 44</td>
<td>0.43 ± 0.08</td>
<td>2323</td>
</tr>
</tbody>
</table>

$^a$Data taken from Ref. (88)

$^b$The truncated form of the enzyme missing the C-terminal tripeptide Cys-Sec-Gly.

$^c$The full length mutant Sec489Cys.

$^d$The WT enzyme produced by semisynthesis.
Figure 10: Structure of the C-terminal Ring motif.
A. Wild-type eight-membered ring. B. Mutant single alanine insertion, creates a eleven-membered ring. C. Mutant double alanine insertion creates a fourteen-membered ring. In the case on the CeTR2 and DmTR enzymes, X = S of Cys, while in mTR3, X = Se of Sec.
Figure 11: Effect of Ring Size on CeTR2 ability to reduce *E. coli* Thioredoxin.
Thioredoxin reductase activity of CeTR2 WT(●), CeTR2-GCACG (□), and CeTR2-GCAACG (◊). Activity is reported in moles of NADPH oxidized per minute per mole of homodimeric enzyme.
Figure 12: Effect of Ring Size on CeTR2 ability to reduce DTNB.
DTNB reductase activity of CeTR2 WT (●), CeTR2-GCACG (□), and CeTR2-GCAACG (◊). Activity is reported in moles of NADPH oxidized per minute per mole of homodimeric enzyme.
Figure 13: Effect of Ring Size on CeTR2 ability to reduce *E. coli* Thioredoxin as a function of pH.

*E. coli* thioredoxin reductase activity of CeTR2 WT (●), CeTR2-GCACG (□), and CeTR2-GCAACG (◊) under varying pH concentrations. Activity is reported as percent maximal activity of moles of NADPH oxidized per minute per mole of homodimeric enzyme.
Chapter 4

Mechanistic Investigation Of C-Terminal Ring Motif

Introduction
To investigate why some TRs utilized Sec and others Cys one must look at the ring-opening step specifically. To study the ring-opening step on the rate of catalysis, I have split TR into two parts. The first part is a C-terminal truncation of TR missing the final eight amino acids (TRΔ8). This C-terminal deletion contains the NADPH binding site, the FAD binding site, as well as the conserved N-terminal dithiol/disulfide. TRΔ8 can be produced as a recombinant protein in E. coli as it has been previously demonstrated that a sixteen amino acid deletion can be made in TR without deleterious effects on protein stability (87). This is verified by TRΔ8 maintaining DTNB reductase activity even though it lacks the C-terminal redox motif. The remaining eight amino acids are produced as a synthetic peptide containing the Cys-Cys dyad in the oxidized form. I have shown that this oxidized peptide is a substrate for the truncated enzyme (72). Thus I can do peptide complementation experiments, which involve adding oxidized C-terminal peptide as the substrate. In this case, the oxidized octapeptide is a substrate for the enzyme because the ring structure must be reduced during the catalytic cycle of the enzyme. This will occur whether the peptide is covalently linked to the C-terminus or not. By removing the oxidized octapeptide from the enzyme, I am able to examine the kinetics of the ring-opening step. The rate at which the disulfides are reduced will be informative with respect to structure-function relationships.
As discussed in Chapter 3, the size of the ring is important for CeTR2 and DmTR catalysis. In addition to exploring the size of the ring in TR catalysis, I want to directly determine the importance of the ring structure in the ring-opening reaction by determining the difference in the rate between cyclic and acyclic forms of the oxidized peptides as shown in Figure 4. The acyclic, oxidized peptide will still have a disulfide bond, but will have unrestricted rotation, unlike the case in which an intervening peptide bond connects the two halves of the disulfide, in the cyclic peptide.

This experiment will be repeated with the other two TRs studied in the Hondal Laboratory, the mouse mitochondrial TR (mTR3) and DmTR. Both of these TRs will also be truncated so that the last eight amino acids are removed from the C-terminus. The CeTR2 enzyme and DmTR do not contain Sec, but rather have a vicinal Cys-Cys dyad at the C-terminus. In studying all three enzymes simultaneously, I can expand the matrix of experiments to be performed so that not only can the kinetic differences between the cyclic and linear forms of the oxidized peptides be studied for each enzyme, but “mutant” peptides can be made in which the penultimate residue can be changed to Cys or Sec so that the effect of leaving group pK$_a$ can be determined for each enzyme. To also examine the effect of leaving group pK$_a$ a peptide containing a mixed disulfide between Cys and TNB, was designed as a low pK$_a$ analog to the Sec peptides, which I expected to display similar activities. A final peptide in which I refer to as the “switch mutant” replaces the Cys-Sec dyad with a Sec-Cys dyad. This construct allows for investigation of which position of the dyad is attacked. This matrix of peptide substrates is shown in Table 10.
Materials and Methods

Materials. DTNB, NADPH, were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) and were of reagent grade or better. All restriction endonucleases, Vent DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA) and used with the supplied buffers according to manufacturer’s guidelines. DNA primers were purchased from IDT (Coralville, IA).

Cloning of Truncated TRs. Previously the cloning of full-length wild type DmTR (65) as well as mutant C-terminal variants (88), have been reported. Following identical cloning conditions from our previous reports, I constructed a truncated DmTR missing the final eight amino acids (PTPASCCS) from the C-terminus and I term this construct DmTR\textsubscript{Δ8}. For the cloning of this construct upstream and downstream primers having respective sequences of 5’- AACACAGATATGCCGCCGTTGGAACCG-3’ and 5’-ACAGCCCGGTACCTTAGTCCAGTCCGGAGCGCTTGGTAGGTGAT -3’ were used to construct the truncated DmTR for production as a recombinant protein in \textit{E. coli} cells. The PCR reaction conditions were described previously in chapter 2, with one notable addition. For the DmTR\textsubscript{Δ8} construct, the final concentration of magnesium chloride was increased to 3mM from 2mM. Without this increase insufficient amplification occurred. The PCR product and plasmid pTYB1 were each digested with Kpn I and Nde I for 2 h at 37 °C, purified and ligated as previously described. The ligated reaction was background digested against Eco RI and transformed into \textit{E. coli} DH5α cells made competent via the Inoue method (79). DNA from isolated colonies were purified using QIAprep Spin
Minprep Kit (Qiagen) and screened by restriction analysis using Nde I and Kpn I, analyzed by analytical 0.8% agarose gel electrophoresis, and positive clones were verified by sequencing of the DNA coding region.

A truncated CeTR2, missing the C-terminal eight amino acids (PRTQGCCG) termed CeTR2Δ8, was cloned using identical amplification conditions to those described in chapter 3 and reported previously (88). The upstream primer of sequence 5′-ACAGCCCCGGATCCCTTCTCATCAAATTTAATTGTCTCTG-3′ incorporates a hexa-histidine tag at the N-terminus and downstream primer of sequence 5′-ACAGCCAGCTTGTCGACGTCTTGTCCATCGGTTTGAAT-3′.

Cloning of truncated mouse TR3, missing the C-terminal eight amino acids (PTVTGCUG) termed mTR3Δ8, was similar to the conditions described above and have been reported by Flemer (89). For this experiment, plasmid pTR3, which contained the full-length sequence of the mouse thioredoxin reductase-3 gene (accession #AF171053), was used as the template DNA. PCR reactions conditions were described in chapter 2, and the upstream primer was of sequence 5′–AACAGACCATGGGAGGGGCAGGCAGAGCTTTG-3′ and the downstream primer was of sequence 5′–ACAGCCAGCTCTTCAGCATCTCGGTATGGAGCTTGGAGAT-3′. The PCR product was then inserted into plasmid pTYB3 using restriction enzymes Nco I and Sap I to produce a plasmid capable of producing the recombinant enzyme in *E. coli* cells. A background digest was performed with Sal I, BSA, and accompanying Sal I Buffer, for 2 hrs at 37°C, to enhance the number of positive clones. Following transformation, purified DNA from isolated
colonies were screened with Nco I and Kpn I. mTR3 contains an internal Kpn I site which yields two DNA fragments of approximately 500bp and 1000bp yielding the desired full length insert product of 1500bp.

Purification of Truncated TRs. For the production of recombinant enzymes CeTRΔ8, DmTRΔ8 and mTR3Δ8, the constructed plasmids were transformed into E.coli ER2566 cells and grown overnight on LB plates containing 200 µg/mL ampicillin. A single isolated colony was inoculated in 100 mL LB media containing 200 µg/mL ampicillin and incubated overnight at 37 °C. The overnight inoculum was pelleted by centrifugation and resuspended in 30 mL of fresh TB media. Three, one-liter pyrex fernbach flasks, each containing TB media containing a final concentration of 200 µg/mL ampicillin and supplemented with 20 mg/L of niacinamide, riboflavin and pyridoxine, were inoculated with 10 mL of resuspended ER25666 E.coli cells. The bacterial growth was incubated at 37 °C, until the O.D. at 600 nm reached 1.0 at which point the cells were incubated on ice until the temperature of the culture reached 20 °C. Protein production was induced by addition of IPTG to a final concentration of 0.5 mM. Cells were grown overnight at 20 °C and then harvested by centrifugation in a Beckman J21B centrifuge (7000 x g, 10 min, 4 °C) and stored frozen at –20 °C.

For the purification of the recombinant enzymes DmTRΔ8 and mTR3Δ8, the frozen cells were thawed on ice and resuspended in 200 mL of 10 mM potassium phosphate, 10 mM NaCl, pH 8.0 and incubated with lysozyme (1mg/mL) at 4 °C for 30 min and followed by brief sonication with a Branson 350 Sonifier (Danbury, CT) to break open any unlysed cells. The cell lysate was cleared by centrifugation in a Beckman
J21B centrifuge (JA-14 rotor) at 7000 xg for 90 min at 4 °C. The clarified supernatant was gravity loaded onto DEAE column (2.5 x 16.5 cm, 80 mL) equilibrated with 10 mM potassium phosphate, 10 mM NaCl, pH 8.0 and washed until the A280 was in the range of 0.10 – 0.05. Protein elution was achieved using a 500 mL linear NaCl gradient of 10 mM-400 mM, in the column buffer. All fractions were analyzed by a Cary 50 UV-vis spectrophotometer from Varian (Walnut Creek, CA) to determine their absorbance at 280 nm and 460 nm. Peak fractions were analyzed by 12% reducing SDS-PAGE and fractions exhibiting A460 and an apparent MW of 55 kDa were pooled and dialyzed into TE (10 mM Tris, 1mM EDTA, pH 8.0) buffer containing 10 mM NaCl. The dialyzed protein was incubated on ice in the presence of 20 mM βME for 30 min, followed by gravity loading onto a column (1.5 x 15 cm, 20 mL) containing 2′,5′-ADP Sepharose 4B (Amersham Biosciences Pharmacia Biotech, Uppsala, Sweden) equilibrated with TE buffer, pH 8.0 with 10 mM NaCl and 20 mM βME. The loaded column was then washed with buffer until the A280 was below 0.5. The protein was liberated from the column with TE buffer containing 3.0 M NaCl. Peak fractions were analyzed and pooled based on their A460 and presence of a 55 kDa band observed on 12% reducing SDS-PAGE. Pooled fractions were gravity loaded onto a Fast Flow 6 Low Substitution Phenyl Sepharose (Amersham Biosciences Pharmacia Biotech, Uppsala, Sweden) column (2.5 x 13 cm, 60 mL) equilibrated with TE buffer, pH 8.0 with 3.0 M NaCl. Following washing with column buffer, elution was carried out using a 500 mL linear gradient of 3.0 M-0.0 M NaCl. Fractions were analyzed for A460 content and visualized by 12% reducing SDS-PAGE, which indicated protein purity greater than 95%. Pooled fractions were dialyzed.
against 2 x 4L of 50mM potassium phosphate, 300mM NaCl, 1mM EDTA at pH 8.0. The dialyzed proteins were concentrated via ultracentrifugation and their concentrations were determined using the $\varepsilon_{460} = 22.6 \text{ mM}^{-1}\text{cm}^{-1}$.

For the purification of recombinant enzyme CeTR2Δ8, ER2566 cells were thawed and then resuspended in 50 mM potassium phosphate, 500 mM NaCl, 10% glycerol, and 10 mM imidazole at pH 8.0 and incubated with lysozyme (1mg/mL) at 4 °C for 30 min and then sonicated briefly. The cell lysate was cleared via centrifugation in a Beckman J21B centrifuge (JA-14 rotor) at 7000 x $g$ for 90 min at 4 °C. The cleared supernatant was incubated with 20 mM βME for 30 min and then gravity loaded onto Ni-NTA agarose (Qiagen, Valencia, CA) column (2.5 x 5.0 cm, 20 mL) equilibrated with 50 mM potassium phosphate, 500 mM NaCl, 10% glycerol, and 10 mM imidazole, pH 8.0 and then washed with 5 column volumes of buffer. The column was washed a second time with an additional 5 column volumes with column buffer containing 20 mM imidazole. Elution of the protein was achieved by addition of buffer containing 250 mM imidazole. Eluted fractions were dialyzed against 50 mM potassium phosphate, 500 mM NaCl, 10% glycerol, and 1 mM dithiothreitol (DTT) pH 8.0, to remove imidazole and maintain a reduced enzyme. Eluent aliquots were concentrated by ultrafiltration using an Amicon Ultra 30000 MWCO (Millipore) concentrator to 1 mL and loaded onto a PD10 desalting column composed of Sephadex™ G-25 medium. This purified enzyme remained in reduced form, and lack the characteristic oxidized 460 peak, making protein concentration determination difficult. The DTNB assay was used to estimate the amount of active enzyme present, by comparing its activity to the WT HisCeTR2. Cloning and
production of full-length wild-type mTR3(72), DmTR(65), and CeTR2 (88) have described previously. In this report the full-length enzymes are respectively abbreviated as mTR3-GCUG, DmTR-SCCS, and CeTR2-GCCG to indicate the sequence of their C-terminal redox active tetrapeptides.

_Synthesis of Peptide Substrates._ The amino acid sequence of cyclic and acyclic octamer peptides was chosen based upon the sequence of the C-terminus of each respective TR used in this study; PTVTGCU for mTR3, PTPASCCS for DmTR, and PRTQGCCG for CeTR2. The procedure for the synthesis of the cyclic and acyclic versions of the C-terminal peptide for mTR3 has been recently published (89) and the corresponding peptides for DmTR and CeTR2 were synthesized using the same procedure. A peptide containing a mixed disulfide between Cys1 of the peptide and 5-thio-2-nitrobenzoate that I termed Pep-TNB (shown in Figure 4), was synthesized for this study. A third type of peptide was synthesized that was of sequence AA1-AA2-AA3-AA4-Sec1-Cys2-AA7-AA8 that I term the “switch peptide” because the position of the Cys and Sec residues have been exchanged. A switch peptide was made for mTR3 and DmTR using the native sequence of the respective enzyme for the remaining residues in the octapeptide. The procedure for the synthesis of the switch peptide was identical to that for cyclic peptides described by Flemer (89).

_DTNB Reductase Activity._ The TRΔ8 constructs were assayed for activity toward DTNB, as described by Arner (70). All assays were performed on a Cary 50 UV-vis spectrophotometer from Varian (Walnut Creek, CA) at 25 °C and pH 7.0 and were
initiated by addition of enzyme. The concentration of homodimeric TR was determined using the flavin extinction coefficient of 22.6 mM$^{-1}\cdot$cm$^{-1}$ (70). Activity was monitored over 2 min with $V_o$ determined from the linear fit. Plots of $V_o/E_T$ versus substrate concentration were fit by the Michaelis-Menten equation using KaleidaGraph 4.02 from Synergy Software (Reading, PA), and activities are reported as moles of NADPH consumed per minute per mole of TR dimer.

The DTNB assay contained 0.2 mM NADPH and 10 mM EDTA in 100 mM potassium phosphate. For each concentration of DTNB, activity was corrected for background by addition of buffer only. Activity was measured by the increase in absorbance at 412 nm, calculated using the extinction coefficient for TNB-S$^-$ (5-thio-2-nitrobenzoate, 13.6 mM$^{-1}\cdot$cm$^{-1}$), and divided by 2 to account for the production of two TNB-S$^-$ molecules per NADPH consumed. The concentrations of enzyme used in the assay were 2 nM of mTR3Δ8, 5 nM of DmTRΔ8, and 5 nM of CeTR2Δ8.

Assays using Peptide Disulfides as Substrates. All lyophilized peptides were weighed and resuspended in a minimal amount of 500 mM potassium phosphate, pH 7.0, to create a working stock solution of peptide in the range of 30 – 60 mM. The peptide complementation assay contained 0-25 mM peptide, 150 µM NADPH, 1 mM EDTA, in 50 mM potassium phosphate at pH 7.0 in a volume of 500 µL. Background activity was corrected for each concentration of peptide by performing an assay in which buffer was added instead of the peptide solution. A second control was performed in the presence of enzyme with peptide missing from the assay. Activity was measured by the decrease in
absorbance at 340 nm for the consumption of NADPH and calculated using an extinction coefficient of 6200 M$^{-1}$•cm$^{-1}$. The concentrations of TRΔ8 and corresponding peptides used in the assay are presented in Table 11. The concentration of each truncated TR was adjusted to achieve a signal to noise ratio that allowed a range of peptide concentrations to be assayed.

When Pep-TNB was used as a substrate the assay conditions were identical to those discussed above except that a wavelength of 412 nm was used to follow the release of the TNB anion over time.

**pH Optima of DTNB Reduction.** Activity toward DTNB was tested as a function of pH for constructs mTR3-GCUG, mTR3Δ8, DmTR-SCCS, and DmTRΔ8. Each assay contained buffer with a final concentration of 100 mM sodium acetate, MES, Tris, and dibasic potassium phosphate adjusted from pH 5.0 to 10.0 in intervals of 0.5 pH units. Each assay contained 150 µM NADPH and 1 mM EDTA. The concentrations of enzyme and DTNB for each of the assays were as follows: 4 nM mTR3-GCUG/2.5 mM DTNB, 3 nM mTR3Δ8/2.5 mM DTNB, 4 nM DmTR-SCCS/1.0 mM DTNB, 5nM DmTRΔ8/5.0 mM DTNB. The activity was measured at 412 nm and background corrected. The data were collected in triplicate, then normalized to the percent of maximal activity for each given construct, and plotted as percent maximal activity versus pH.

**pH Optima of Peptide Reduction.** The truncated TR enzymes were tested for their ability to reduce their cognate C-terminal peptides as a function of pH. The final 500 µL reaction contained 100 mM each of MES, sodium acetate, potassium phosphate (dibasic),
and Tris, 1 mM EDTA, 0.150 mM NADPH. Concentrations of the enzymes and peptides assayed were as follows: 10 nM mTR3Δ8/2 mM cyclic PTVTGCUG, 50nM mTR3Δ8/2 mM acyclic PTVTCGUG, 15nM mTR3Δ8/0.5 mM acyclic PTVTGC-TNB, 50nM DmTRΔ8/1 mM acyclic PTPASCUS, and 25nM DmTRΔ8/0.5 mM acyclic PTPASC-TNB. Activity was monitored for changes in A340/min or A412/min as described above. Each pH point was assayed in duplicate or triplicate and were background corrected against the spontaneous reduction of NADPH at acidic pH.

Results

Purification of Enzymes. mTRΔ8 and DmTRΔ8 proteins were purified using three different chromatographic steps to homogeneity as judged by SDS-PAGE as shown in Figure 14. Protein yields for mTRΔ8 and DmTRΔ8 were 32 mg/L and 156 mg/L of culture, respectively. The clone for CeTR2Δ8 contained a hexa-histidine tag, which allowed for a single step purification by an affinity column and had a similar purity as the other TRs in this study as judged by SDS-PAGE (Figure 14). The protein yield for CeTR2Δ8 was 35 mg/L of cell culture.

DTNB Reductase Activity. In this study I created truncated TRs missing their final eight amino acids and evaluated their ability to reduce DTNB. A summary of the kinetic data is presented in Table 12. All truncated enzymes maintained their ability to reduce DTNB at comparable rates compared to their corresponding WT enzymes. In the case of mTR3Δ8, its $k_{cat}$ increased 2.3-fold, while $K_m$ also increased 1.8-fold, resulting in nearly identical catalytic efficiency compared to its WT enzyme. DmTRΔ8
followed a similar pattern, its $k_{\text{cat}}$ increased 8.2-fold, while its $K_{m}$ increased 18.6-fold, resulting in a decrease in catalytic efficiency of 2.3-fold. The kinetic constants for CeTRΔ8 were essentially the same as its full-length enzyme.

Assays using Peptide Substrates. During the catalytic cycle electrons are transferred from the N-terminal disulfide redox center to the C-terminal disulfide/selenylsulfide redox center. Truncated TRs are capable of reducing peptide disulfides/selenylsulfides that correspond to their missing C-terminal sequence (ring opening step – Figure 15) (65, 72) (89). Using this property of TR, I was able to evaluate the relative importance that the unique eight-membered ring makes to the catalytic rate. This is accomplished by comparing the turnover rate of cyclic and acyclic peptide disulfide/selenylsulfide substrates. In each instance the WT amino acid sequence was oxidized creating a disulfide/selenylsulfide linkage, resulting in the formation of a cyclic intrachain or an acyclic interchain peptide. The cyclic intrachain disulfide/selenylsulfide results in the formation of a strained eight-membered ring, while the interchain disulfide/selenylsulfide linkage results in an acyclic peptide lacking this strained eight-membered ring. One important feature of this study is to compare the importance of the ring structure between Sec-containing and Cys-containing TRs. Thus for a Sec-containing TR the relevant comparative rates are for the cyclic Se-peptide and the acyclic Se-peptide ($X = \text{Se}$ in Figure 4), and for a Cys-containing TR the relevant rates are the cyclic S-peptide and the acyclic S-peptide ($X = \text{S}$ in Figure 4). The second order rate constants for these peptide turnover rates are given in Table 13 (Kinetic plots for which these rates are derived are provided in Figure 16, Figure 17, and Figure
and the important ratios for comparison are summarized in Table 14. The data in Table 14 indicate that the eight-membered ring contributes moderately to the catalytic rate for the Sec-containing mouse enzyme since the ratio of cyclic to acyclic peptides is 32, however the data indicates that the eight-membered ring is of much greater importance for the Cys-containing TRs from *D. melanogaster* and *C. elegans* as the ratio of turnover rates for cyclic and acyclic peptides is 1025 and 2267, respectively.

“Mutant” peptides were also constructed in which the penultimate residue was changed from Sec to Cys (mTR3) or Cys to Sec (DmTR, CeTR2) for both the cyclic and acyclic peptides. These peptides were assayed as substrates for their respective enzymes. The results given in Table 13 indicate that S for Se substitution has dramatic effects on the catalytic rate for both the cyclic and acyclic peptides in the case of the mammalian enzyme (8667-fold lower and 410-fold lower, respectively). Conversely, Se for S substitution improves the turnover rate by 12-fold in the case of DmTR (compare 41 to 499) or only moderately decreases the rate by 2.8-fold in the case of CeTR2. Interestingly, the acyclic Se-peptide has comparable activities to the cyclic S-peptide in the cases of DmTR and CeTR2. While the acyclic Se-peptide has moderately lower activity than the cyclic Se-peptide in the case of the mammalian enzyme, the acyclic Se-peptide has much higher activity than the cyclic S-peptide (167-fold) with mTR3. This indicates that the presence of a selenium atom in the peptide contributes much more to the rate than does the presence of the ring in the case of the mammalian enzyme. In the case of the Cys-containing TRs, the selenium atom in the penultimate position makes a near equal contribution to the rate in comparison to the ring since the activity of the
acyclic Se-peptide has a similar rate to the cyclic S-peptide. These two facts are supportive of a strong leaving group effect in the ring opening reaction. This hypothesis is supported by the data using Pep-TNB as a substrate for all three enzymes. This substrate contains a highly reactive mixed disulfide bond due to the presence of the 5-thio-2-nitrobenzoate group. The sulfur atom of this moiety has a $pK_a$ of 4.75 (90), comparable to that of a selenol (5.2, (91)). The assay results using this substrate indicate that it has similar activity to the cyclic, WT peptide for each respective enzyme. For example, the turnover rate for Pep-TNB is 220 min$^{-1}$mM$^{-1}$ for mTR3 while the cyclic Se-peptide has a turnover rate of 260 min$^{-1}$mM$^{-1}$ (Table 13). Similar results are shown for the Cys-containing TRs in Table 13. Further strength of this leaving group hypothesis is given by the ratio of acyclic Se-peptide/acyclic S-peptide turnover rate for all three enzymes listed in Table 14. This ratio is of similar magnitude in all three cases indicating the importance of the lower $pK_a$ selenol in comparison to that of the thiol (5.2 vs. 8.3).

Additionally a “switch” mutant was constructed in which a cyclic selenylsulfide linkage was made, however the positions of Sec and Cys were “switched” so that Sec was in the first position of the dyad. The mammalian enzyme had an activity of 0.23 min$^{-1}$mM$^{-1}$ using this peptide substrate, which is 1130-fold lower than the cyclic peptide in which Sec is in the second position of the dyad (260 min$^{-1}$mM$^{-1}$). In comparison DmTR had an activity of 4.6 min$^{-1}$mM$^{-1}$ using this switch peptide, which is a 109-fold drop in comparison to the peptide with Sec in the second position of the dyad (499 min$^{-1}$mM$^{-1}$). The lower effect of the switch mutant on the Cys-containing TR is likely due to the fact
that a sulfur atom must be in the leaving group position when this ring is open since it contains a Cys₁-Cys₂ dyad. Thus the drop in activity for DmTR using the switch peptide as substrate must be due to the fact that the sulfur atom of CysIC must attack a more electronegative atom. The effect of this mutation is illustrated in Error! Reference source not found.. This result strongly argues for the second residue of the dyad (either Cys₂ or Sec₂) to be in the leaving group position during the ring opening step.

**pH Optima of DTNB Reduction.** To evaluate the pH dependence of the reduction of DTNB in the truncated enzymes and their full-length WT counterparts, I performed pH rate profiles (Activities are indicated in Table 15). The truncated mouse enzyme shows a pH optimum for the reduction of DTNB at pH 6.5, while truncated DmTR has a pH optimum at one pH unit higher as shown in Figure 20A. For comparison the pH rate profiles for the full-length enzymes are shown in Figure 20B. For the full-length mouse enzyme the pH optimum is shifted to 7.0-7.5. The full-length enzyme from *D. melanogaster* also showed a basic pH shift to pH 8.5. The results indicate that the C-terminal redox center causes a shift in the pH optimum for both enzymes.

**pH Optima of Peptide Reduction.** The pH dependence for the reduction of peptides containing Sec or a mixed disulfide with TNB were assayed with their corresponding truncated TR (Figure 21, Table 16). Figure 21A shows pH rate profiles comparing activity of mTR3Δ8 with acyclic PTVTGCU and DmTRAΔ8 with acyclic PTPASCUS and their pH optima’s were determined to be 7.9 and 8.4, respectively. The cyclic PTVTGCU peptide had comparable pH optima of 8.4 with mTR3Δ8 (data not
shown). The pH dependence of the reduction of the acyclic peptides PTVTGC-TNB and PTPASC-TNB with their cognate truncated enzymes is shown in Figure 21B. The mTR3Δ8 enzyme has a pH optima of 6.0, while the DmTRΔ8 pH optima shifts ca. 1.5-2.0 units, to between 7.4-7.9. It was observed at pH 6.0 and to a lesser extent pH 6.5, the A412/min with PTVTGC-TNB deviated from linearity, showing a initial burst and then lagging. I believe that the enzymes CysIC is forming a stable mixed disulfide with PTVTGC and the initial burst can be contributed to the initial reaction of peptide and with TR resulting release of TNB-S’. This however was not a stiochiometric event indicating there was turnover following initial burst phase, just severely impaired.

**Conclusions**

The data presented here in this chapter reveals details into the mechanistic differences between the two classes of TR. The truncated TRs were purified and exhibited high DTNB reductase activities. The ability for the truncated enzymes to turnover DTNB is excellent evidence for having correctly folded and functional enzyme. This allowed for more elaborate experiments using cyclic and acyclic peptides that mimic the removed C-terminal sequence, to investigate the mechanism of “ring opening” between the vicinal Cys1-Cys2 or Cys1-Sec2 dyads.

These experiments allow one to conclude that, firstly, based on the activities of the ratios of the WT cyclic peptides vs. the WT acyclic peptides, the eight-membered ring structure is much more important for the Cys-containing class of TRs (CeTR2, DmTR) than to the Sec-containing member, mTR3. Second, for mTR3, the Se atom is much more important than the ring structure. This comes from the fact that the acyclic Sec
peptide had 273-fold more activity than the cyclic Cys peptide. For the Cys-containing TRs, the cyclic Cys peptide had higher rates than the acyclic Sec peptides though were of similar magnitudes (1.3 and 7.2-fold decreases in activity). These results suggest that the Cys₁-Cys₂ ring is important for enhancing Cys₂ ability to act as a better leaving group during ring opening, while Sec does not need the ring structure because it low pKₐ makes it a good leaving group. This is supported by the rates determined for the acyclic Peptide-TNB construct. The TNB group, utilizes a S atom, however is a much better leaving group than Cys, due to its lower pKₐ. This peptide behaves similarly to the acyclic Sec peptides, and restores activity to near the WT cyclic peptides rates. Lastly, the poor activity of “switch” mutant peptides, reveal that position 1 of the dyad must be attacked by the Cys₁₇ residue during ring opening.

To examine the mechanism further, pH profiles comparing the activities of the truncated mTR3 and DmTR enzymes with DTNB, acyclic Sec peptides and their Pep-TNB construct. The results indicate that there are differences between the two enzymes ability to reduce these substrates. The mTR3Δ8 is capable of reducing these substrates at lower pH compared to DmTR which has implications for mechanistic differences which will be discussed in chapter 5.
Table 10: Summary of Peptide Complementation Experiments to be Performed Between Truncated Enzyme and Cognate, Oxidized Peptide.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th></th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>CeTR2Δ8</td>
<td>DmTRΔ8</td>
<td>mTR3Δ8</td>
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<tr>
<td>WT acyclic peptide</td>
<td>PRTQGCG</td>
<td>PTPASCSC</td>
<td>PTVTGCUG</td>
</tr>
<tr>
<td>WT cyclic peptide</td>
<td>PRTQGCCG</td>
<td>PTPASCSCS</td>
<td>PTVTGCUG</td>
</tr>
<tr>
<td>Mutant acyclic peptide</td>
<td>PRTQGCUG</td>
<td>PTPASCUS</td>
<td>PTVTGCUG</td>
</tr>
<tr>
<td>Mutant cyclic peptide</td>
<td>PRTQGCUG</td>
<td>PTPASCUS</td>
<td>PTVTGCCG</td>
</tr>
<tr>
<td>Acyclic TNB peptide</td>
<td>PRTQGC TNB</td>
<td>PTPASC TNB</td>
<td>PTVTGC TNB</td>
</tr>
<tr>
<td>Cyclic “switch” peptide</td>
<td>Not Synthesized</td>
<td>PTPASCUS</td>
<td>PTVTGUCG</td>
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</tbody>
</table>

*For each truncated TR shown above, each will be assayed with its cognate, oxidized peptide in the cyclic and acyclic form. In addition, each truncated enzyme can be assayed with the cyclic and acyclic forms of the peptide in which the penultimate amino acid is replaced with Sec or Cys (depending on the original WT sequence).*
Table 11: Truncated TR and Peptide Concentrations used in Peptide Complementation Experiments.

<table>
<thead>
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<th>Peptide</th>
<th>Concentration Range</th>
<th>Enzyme</th>
<th>Concentration</th>
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<tr>
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</tr>
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<td>1000nM</td>
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<td>DmTR-Δ8</td>
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<tr>
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<td>DmTR-Δ8</td>
<td>2000nM</td>
</tr>
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<td>Cyclic PTPASCUS</td>
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<td>Cyclic PRTQGC-TNB</td>
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<td>CeTR2-Δ8</td>
<td>20nM</td>
</tr>
<tr>
<td>Enzyme</td>
<td>$k_{\text{cat}}$ (min$^{-1}$)</td>
<td>$K_m$ (mM$^{-1}$)</td>
<td>$k_{\text{cat}}/K_m$ (min$^{-1}$•mM$^{-1}$)</td>
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<tr>
<td>------------------</td>
<td>-------------------------------</td>
<td>------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>mTR3-G$\text{CUG}^a$</td>
<td>1251 ± 71</td>
<td>0.45 ± 0.09</td>
<td>2720</td>
</tr>
<tr>
<td>mTR3-$\Delta$8</td>
<td>2905 ± 232</td>
<td>0.83 ± 0.27</td>
<td>3500</td>
</tr>
<tr>
<td>DmTR-$\text{SCCS}^a$</td>
<td>157 ± 12</td>
<td>0.22 ± 0.07</td>
<td>713</td>
</tr>
<tr>
<td>DmTR-$\Delta$8</td>
<td>1294 ± 277</td>
<td>4.1 ± 2.1</td>
<td>316</td>
</tr>
<tr>
<td>CeTR2-G$\text{CCG}^b$</td>
<td>134 ± 7</td>
<td>0.41 ± 0.09</td>
<td>327</td>
</tr>
<tr>
<td>CeTR2-$\Delta$8</td>
<td>152 ± 14</td>
<td>0.53 ± 0.19</td>
<td>287</td>
</tr>
</tbody>
</table>

$^a$Reported in ref. (88).
$^b$Reported in supplemental of ref. (88).
Table 13: Rate Data for Truncated TRs using Peptide Disulfides as Substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme (octapeptide)</th>
<th>Rate (min(^{-1})•mM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mTR3(\Delta 8)</td>
<td>DmTR(\Delta 8)</td>
</tr>
<tr>
<td></td>
<td>(PTVTG\textit{CuG})</td>
<td>(PTPAS\textit{CCS})</td>
</tr>
<tr>
<td>Cyclic \textit{Se}-peptide</td>
<td>260 ± 44(^a)</td>
<td>499 ± 24</td>
</tr>
<tr>
<td>Acyclic \textit{Se}-peptide</td>
<td>8.2 ± 1.4 (^a)</td>
<td>31 ± 0.4</td>
</tr>
<tr>
<td>Cyclic \textit{S}-peptide</td>
<td>0.03 ± 0.003(^a)</td>
<td>41 ± 1.5</td>
</tr>
<tr>
<td>Acyclic \textit{S}-peptide</td>
<td>0.02 ± 0.001(^a)</td>
<td>0.04 ± 0.001</td>
</tr>
<tr>
<td>Cyclic “Switch” peptide</td>
<td>0.23 ± 0.012</td>
<td>4.6 ± 0.04</td>
</tr>
<tr>
<td>DTNB</td>
<td>3500 ± 578(^a)</td>
<td>316 ± 79</td>
</tr>
<tr>
<td>Peptide-TNB</td>
<td>220 ± 62</td>
<td>21 ± 0.45</td>
</tr>
</tbody>
</table>

\(^a\)From Ref. (89)

\(^b\)ND = Not determined
Table 14: Ratio of Turnover Rates of Peptide Substrates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cyclic Se/Cyclic-S</th>
<th>Acyclic-Se/Acyclic-S</th>
<th>Cyclic WT/Acyclic WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTR3Δ8</td>
<td>8667</td>
<td>410</td>
<td>32</td>
</tr>
<tr>
<td>DmTRΔ8</td>
<td>12</td>
<td>775</td>
<td>1025</td>
</tr>
<tr>
<td>CeTR2Δ8</td>
<td>0.4</td>
<td>317</td>
<td>2267</td>
</tr>
</tbody>
</table>
### Table 15: pH Dependence of Reduction of DTNB

<table>
<thead>
<tr>
<th>pH</th>
<th>mTR3-GCU</th>
<th>mTR3Δ8</th>
<th>DmTR-SCCS</th>
<th>DmTRΔ8</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>8.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.58</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5.5</td>
<td>32.48</td>
<td>70.26</td>
<td>10.72</td>
<td>ND</td>
</tr>
<tr>
<td>6.0</td>
<td>422.49</td>
<td>935.87</td>
<td>48.41</td>
<td>259.80</td>
</tr>
<tr>
<td>6.5</td>
<td>945.16</td>
<td>1570.67</td>
<td>189.34</td>
<td>612.01</td>
</tr>
<tr>
<td>7.0</td>
<td>1056.68</td>
<td>1409.72</td>
<td>216.30</td>
<td>797.06</td>
</tr>
<tr>
<td>7.5</td>
<td>1062.19</td>
<td>1149.10</td>
<td>226.41</td>
<td>974.02</td>
</tr>
<tr>
<td>8.0</td>
<td>849.88</td>
<td>810.05</td>
<td>233.46</td>
<td>900.25</td>
</tr>
<tr>
<td>8.5</td>
<td>733.15</td>
<td>383.17</td>
<td>246.63</td>
<td>800.00</td>
</tr>
<tr>
<td>9.0</td>
<td>617.03</td>
<td>330.47</td>
<td>205.58</td>
<td>570.83</td>
</tr>
<tr>
<td>9.5</td>
<td>481.62</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>168.50</td>
<td>313.73</td>
</tr>
<tr>
<td>10.0</td>
<td>368.57</td>
<td>ND</td>
<td>98.65</td>
<td>123.04</td>
</tr>
</tbody>
</table>

<sup>a</sup>Activities reported as moles of NADPH oxidized/min/mole TR.

<sup>b</sup>ND = Not Determined
Table 16: pH Dependence of Reduction of Peptides Substrates

<table>
<thead>
<tr>
<th>pH</th>
<th>Cyclic PTVTGCUGA</th>
<th>Acyclic PTVTGCUGA</th>
<th>Acyclic PTVTGCTNB</th>
<th>Acyclic PTPASCUSb</th>
<th>Acyclic PTPASC-TNBb</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>69.13c</td>
<td>17.04</td>
<td>1.72</td>
<td>0.64</td>
<td>NDd</td>
</tr>
<tr>
<td>6.0</td>
<td>83.60</td>
<td>21.22</td>
<td>59.80</td>
<td>4.18</td>
<td>10.29</td>
</tr>
<tr>
<td>6.5</td>
<td>114.15</td>
<td>32.48</td>
<td>134.64</td>
<td>11.25</td>
<td>20.01</td>
</tr>
<tr>
<td>7.0</td>
<td>173.63</td>
<td>41.80</td>
<td>138.89</td>
<td>18.65</td>
<td>24.51</td>
</tr>
<tr>
<td>7.4</td>
<td>223.47</td>
<td>63.99</td>
<td>125.16</td>
<td>29.58</td>
<td>26.86</td>
</tr>
<tr>
<td>7.9</td>
<td>239.55</td>
<td>67.20</td>
<td>113.40</td>
<td>29.26</td>
<td>26.72</td>
</tr>
<tr>
<td>8.4</td>
<td>252.41</td>
<td>65.59</td>
<td>89.05</td>
<td>35.05</td>
<td>20.81</td>
</tr>
<tr>
<td>8.9</td>
<td>205.79</td>
<td>61.74</td>
<td>59.80</td>
<td>24.44</td>
<td>12.99</td>
</tr>
<tr>
<td>9.4</td>
<td>176.85</td>
<td>58.52</td>
<td>37.91</td>
<td>22.19</td>
<td>7.84</td>
</tr>
<tr>
<td>9.8</td>
<td>146.30</td>
<td>51.77</td>
<td>23.53</td>
<td>16.40</td>
<td>5.39</td>
</tr>
</tbody>
</table>

aAssayed with mTR3Δ8.
bAssayed with DmTRΔ8.
cActivity is reported as moles of NADPH oxidized/min/mole TR.
dND = Not Determined.
Figure 14: SDS-PAGE of Purified TRs
A 12% reducing SDS-PAGE showing purity of enzymes used in this study. Lane 1, TriChromRanger™ prestained protein molecular weight marker mix, Lot:IE0014 (Pierce, Rockford, IL), lane 2, HisCeTR2, lane 3, HisCeTR2Δ8, lane 4, DmTR, lane 5, DmTRΔ8, lane 6, semi-synthetic mTR3, lane 7, mTR3Δ8.
Figure 15: TR has a conserved enzymatic mechanism.

A.) Full length TR accepts reducing equivalents from NADPH, which transfers a hydride to a non-covalently bound FAD cofactor. The N-terminal redox active center is reduced to form a charge transfer complex (FADH−SHCT), while the interchange Cys (SHIC) attacks the C-terminal redox center of the neighboring subunit (designated with prime (′) and shown in red), which I refer to as the “ring opening step”. Following this “ring opening step”, the nucleophile (NucX) forms a mixed disulfide with Trx and is resolved by the vicinal Cys (ResSH) to reform the eight member ring.  

B.) To determine the contribution the “ring opening step” has to the rate of Trx reduction, I constructed a truncated TR lacking the C-terminal eight residues. Cyclic and acyclic peptides (red) (see Figure 4) corresponding to the C-terminal sequence can be reduced by the truncated enzyme, and a rate determined (see Table 13). 

C.) GR is in essence a truncated TR missing its C-terminal sixteen residues and GSSG (red) is analogous to the TR C-terminal redox center. 

D.) Truncated TR is incapable of reducing linear disulfide substrates such as GSSG or cystine, whiles the highly reactive disulfide DTNB (red), and is readily reducible.
Figure 16: Peptide Complementation Kinetic Plots of CeTRΔ8.
A. Cyclic PRTQGCCG B. Acyclic PRTQGCCG. C. Cyclic PRTQGCUG. D. Acyclic PRTQGCUG. E. Acyclic PRTQGC-TNB.
Figure 17: Peptide Complementation Kinetic Plots of DmTRA8
A. Cyclic PTPASCCS. B. Acyclic PTPASCCS. C. Cyclic PTPASCUS. D. Acyclic PTPASCUS. E. Acyclic PTPASC-TNB. F. Cyclic PTPASUCS.
Figure 18: Peptide Complementation Kinetic Plots of mTR3Δ8.
A. Cyclic PTVTGCUG. B. Acyclic PTVTGCUG. C. Cyclic PTVTGCCG. D. Acyclic PTVTGCCG. E. Acyclic PTVTGC-TNB. F. Cyclic PTVTGUCG.
Figure 19: Effect of selenium on “ring opening”
A.) In the wild type case, the eight-membered ring is composed of the Cys$_1$-Sec$_2$ dyad. The interchange thiolate attacks the electrophilic S of Cys$_1$ allowing the Se of Sec$_2$ to act as the leaving group. B.) The “switch” mutant peptide eight-membered ring is composed of the Sec$_1$-Cys$_2$ dyad. In this instance, the interchange thiolate attacks the more electronegative Se atom of Sec$_1$, which is less reactive and forces the poorer leaving group S of Cys$_2$ to leave.
Figure 20: Activity toward DTNB as a function of pH for Thioredoxin Reductase. 
A.) Truncated mTR3Δ8 (■) and truncated DmTRΔ8 (○). B.) Full length mouse wild type enzyme mTR3-GCUG (■), full length wild type DmTR-SCCS (○). Results are reported as a percentage of the maximal activity for each plot.
Figure 21: Activity of truncated TR toward peptide substrates as a function of pH.
A.) Truncated mTRΔ8 assayed with acyclic PTVTGCU (■) and truncated DmTRΔ8 assayed with acyclic PTPASCUS (○). B.) Truncated mTRΔ8 assayed with acyclic PTVTG-C-TNB (■) and truncated DmTRΔ8 assayed with acyclic PTPASC-TNB (○). Results are reported as a percentage of the maximal activity for each plot.
Chapter 5

Proposed Mechanism

Discussion

A key question in seleno-enzymology is why certain enzymes have evolved the rare and unique Sec residue which requires intricate recoding machinery for interpreting the UGA opal stop codon as a sense codon for Sec (92, 93). In the case of TR, it had always been assumed that the selenium atom was required for efficient nucleophilic attack on the disulfide bond of Trx, but this notion was largely dispelled by Kanzok and coworkers who showed that DmTR has very high activity towards its cognate Trx as well as the *E. coli* substrate (25). Further support for the unimportance of Se in the reaction was given in chapter 2, showing that the CeTR2 is also capable of utilizing Cys to reduce *E.coli* Trx, even with out the aid of flanking Serine residues (26). The work presented here builds on our previous work, which put forth the hypothesis that the Sec residue is required in the ring opening step of the reaction for the mammalian enzyme. I summarize the evidence for this hypothesis below.

Previously, Eckenroth has demonstrated that a truncated TRΔ3 could be used to reduce oxidized tetrapeptides, corresponding to the wild type and mutant C-terminal sequence of mTR3 (GCUG, GCCG) and DmTR (SCCS, SCUS), respectively. Evaluation of the ratio of rates of the ring opening step for Se/S containing peptides indicated that the Se atom was more important to mTR3 (308-fold rate reduction) enzyme than it was for DmTR (5.65-fold rate reduction) enzyme (65).
When the C-terminal vicinal Cys$_1$Cys$_2$ and Cys$_1$Sec$_2$ residues are oxidized, a strained eight-membered ring is formed. NMR structural determinations of tetrapeptides indicate that Cys$_1$Cys$_2$ peptides undergo cis/trans equilibrium, while the Cys$_1$Sec$_2$ peptide is predominately forced into a trans conformation (Deker, unpublished work). To investigate the importance of the ring to catalysis, substrate docking studies predicted that the Cys$_1$Cys$_2$ containing enzymes ability to switch conformations of the ring is necessary, indicating that upon reduction of the cis conformation, Cys$_2$ is in position for proton extraction from a conserved histidine, stabilizing the Cys$_2$ thiolate enhancing its ability to act as a good leaving group. Conversely, proton extraction is not possible when Sec$_2$ is in the trans conformation, however it is not necessary since the selenolate anion is a better leaving group than the thiolate because of its lower pK$_a$.

These observations led us to investigate the importance of the eight-membered ring in the catalytic cycle. As stated in chapter 3, this hypothesis was evaluated by constructing mutant enzymes possessing one or two alanine residues inserted between the vicinal Cys$_1$Cys$_2$/Sec$_2$ dyads of mTR3, DmTR, and CeTR2. These insertions had the effect of expanding the ring size (eleven and fourteen vs. eight-membered rings) and relieving ring strain. By expanding the ring size, Cys$_2$/Sec$_2$ becomes separated away from the conserved histidine, resulting in loss of activity. The effect of these single and double alanine insertions on the reduction of *E.coli* Trx indeed showed a decrease in rate compared to the WT rate of 4-6-fold, 150-300-fold, and 90-145-fold for mTR3, DmTR, and CeTR2, respectively. The fact that mTR3 insertions resulted in only a modest decrease in activity while both DmTR and CeTR2 had more dramatic losses in activity is
strong evidence in favor of our hypothesis that the ring structure is more important for the Cys containing TRs, than the Sec containing mTR3.

In this study I also considered why some TRs utilize Sec, while others exploit Cys, during their catalytic cycle. To investigate this, peptide complementation was used. This technique takes advantage of the ability of truncated TRs to reduce cyclic and acyclic octapeptides, corresponding to their missing C-terminal sequences. A comparison of the catalytic efficiencies of peptides containing either sulfur or selenium, in the penultimate position, provides insights into their roles in the ring opening process. The ratios of rates of the ring opening step for cyclic Se/cyclic S containing peptides verified that the Se atom was more important to mTR3 (8667-fold) enzyme than it was for DmTR (12-fold) and CeTR2 (0.36-fold) enzymes. Additionally, comparisons of the ratios for the rates of the WT cyclic/acyclic peptides reveal that, for mTR3 (32-fold loss in activity), the ring structure is not as important for activity, while in the case of the Cys-containing enzymes DmTR (1025-fold loss in activity) and CeTR2 (2267-fold loss in activity), a large decrease in activity demonstrates that the eight-membered ring is important for catalysis. I believe that this result supports that the eight-membered ring must be pertinent for aligning Cys2 with the conserved histidine to allow for protonation of the leaving group, thus for the Cys class of TRs, it is the ring that allows for optimal activity when utilizing a S atom. For mTR3, the Se atom is necessary for activity, and its presence is more important that the ring structure. This is demonstrated by the acyclic Se peptide having 273-fold more activity than with the cyclic S peptide (compare 8.2 min⁻¹ • mM⁻¹ with 0.03 min⁻¹ • mM⁻¹). All three truncated enzymes were also assayed with
acyclic peptides containing Cys and Sec in the penultimate position. By comparing the ratios of the rates for these peptides, the effect of leaving group chemistry on the reaction can be evaluated. Sec is a better leaving group than Cys because of its low pK_a. This is observed by acyclic Sec peptides being more reactive than the acyclic Cys peptides. A comparison of these results reveal similar differences in rate loss among the three enzymes: mTR3Δ8 (410-fold loss), DmTRΔ8 (775-fold loss) and CeTR2Δ8 (317-fold loss). This result supports that the leaving group effect is the predominate driving force resulting in activity. Thus for mTR3, the incorporation of a Se atom is essential for maintaining a good leaving group, whereas for the Cys-containing TRs, the eight-membered ring and its proximity to the critical acid catalyst histidine, allows the S atom to act a good leaving group in a similar matter to Se. This is illustrated in Figure 22.

To verify this observation the truncated enzymes were assayed for activity with an acyclic C-terminal peptide analog conjugated with a mixed disulfide with the highly reactive, low pK_a, TNB compound. This resulted in rescuing activity of the Cys-containing peptides to comparable levels with the Sec-containing peptides. The ratios of Pep-TNB/acyclic S-peptides show fold-rate enhancements of 11000, 525 and 533 for mTR3, DmTR and CeTR2, respectively. The substitution of a better leaving group like TNB in place of Cys, resulted in restoring the lost activity to a comparable rate similar to the acyclic Se peptides. These results add to the support that the leaving group is important.
The poor activities of the cyclic “switch” mutants (Sec$_1$-Cys$_2$ dyad), indicate that the AA$_1$ (of the dyad) position must be involved in the attack from the interchange Cys$_{IC}$ during the ring opening step.

The results of enlarging the ring and the ratio of the peptide complementation rates of the WT cyclic/acyclic peptides indicate that the ring structure is of only moderate importance for the Sec containing mTR3, while it is essential for the Cys contain DmTR and CeTR2. The results of the investigation into the role of Sec or Cys in the ring opening step indicate that their role is involved as the leaving group. For the Cys containing TRs, the leaving group is very important. The ring structure makes Cys a better leaving group by positioning it for protonation by a conserved histidine that acts a general acid. When the ring is disrupted, as in the acyclic S-peptide, the leaving group is disengaged from the general acid, which prevents protonation, and results in extremely poor activity. The activity can be restored by substituting Cys with a good leaving group like Sec or TNB. For the Sec-containing TR, the Sec leaving group is very important, indicated by the severe loss in activity when a Cys residue was substituted in its place and restored by the TNB moiety. Also the “switch” mutant results indicate the Sec must be in the second position of the dyad to be active. The data indicates that the Sec containing ring disconnects the leaving group from the general acid, however, because of Sec low p$K_a$ results in the selenolate, which can overcome the disconnection.

The data suggests that for the Cys TRs, are two questions are related. The ring is important for positioning the leaving group Cys near the His, which makes Cys a good
leaving group and for the Sec TR the ring is of less importance because it contains a low pK_a leaving group and does not require protonation.

This investigation indicates that the differences in the two classes of TRs mechanism occur in the ring opening step. Why does the Sec TR need a low pK_a leaving group? The pH rate profile data with the truncated enzymes indicated distinct pH optima between mTR3 and DmTR. Of these enzymes, the full length and truncated forms of each were characterized for their pH dependence for the reduction of the small molecule DTNB. Addition of the C-terminal redox active site, in the full length enzymes, caused a shift in the optimal pH toward the alkaline. It has been shown that the pK_a’s for the Cys_CT (4.8), Cys_IC (6.2-7.5) and His (9.2) acid/base catalyst of yeast GR could shift dramatically, from the usually expected values (94-96). Since the truncated TRs used in this study resemble GR, it is suitable to use the pK_a values of yeast GR since there is high degree of homology among the active site titratable groups. In this reaction the Cys_IC thiolate needs to be stabilized by the proton on His before it can attack the S atom of DTNB. The stabilization of the thiolate would be rate limiting step. The Cys_CT residue would be ionized and capable of resolving the formed mixed disulfide between Cys_IC and TNB completing the enzymatic cycle. In the case of the full length WT TRs, DTNB is reduced either by the N-terminal or C-terminal redox center. The histidine residue would still be critical for stabilizing the Cys_IC thiolate for attack either on the C-terminus or DTNB substrate.

To look at the effect Sec has on the protonation events occurring in the active site, I performed a similar experiment as with DTNB, but replaced the substrate with acyclic
Sec containing peptides. I expected similar results as obtained through the DTNB data. However, instead I observed a pH shift toward the alkaline. A possible explanation for this is that the $pK_a$ of Sec is not as close to the $pK_a$ of the TNB-S$^-$ (5.2 vs 4.75) as indicated in the literature. It is known that the $pK_a$ of a free amino acid can vary upon its incorporation into a peptide and protein sulfhydryl group $pK_a$ values can be very different.

Additionally, Pep-TNB substrates were designed thinking they would mimic the activity of the acyclic Sec peptides. This assumption correlated well with the DmTRΔ8 enzyme, however, Figure 21B shows that the mTR3Δ8 enzyme pH optimum was lowered 1.5-2.0 units compared to the DmTRΔ8 enzyme. Why might this be? First, as stated above the Sec residue $pK_a$ must be higher than that of the reported for free Sec. Second, the $pK_a$ of the Cys$_{CT}$ thiol must be lower in the mTR3 enzyme while DmTR must be elevated in comparison to each other. This property would allow for the ring to be opened at lower pH in the case of the mammalian enzyme.

Since the truncated TRs used in this study resemble GR, it is suitable to use the $pK_a$ values of yeast GR since there is high degree of homology among the active site titratable groups. An area for future study would be to determine the $pK_a$ of the truncated TR enzymes and compare their values to the GR. It was demonstrated that a human TRΔ16 could not regain GR activity (87). The environment surrounding the active site pocket was negatively charged compared to the positively charged GR active site. This undoubtedly would have an effect upon the TR active site $pK_a$’s.
There are several implications for mTR3’s ability to function at low pH. One possibility is for the role in redox signaling as suggested by Sun et al (77). When hydrogen peroxide levels rise in the cell, TR becomes oxidized. Upon accumulation of oxidized TR, cellular targets remain oxidized. The ensuing build up of reactive oxygen species, can cause activation of redox regulating pathways such as the through apoptosis signal-regulating kinase 1 (97). Second, the cellular milieu contains several small molecules (hydrogen peroxide (59), dehydroascorbate (98), selenodiglutathione (99)), that have almost exclusively be shown to be capable of reduction by mTR3. The ability of mTR3 to still form a charge transfer complex at low pH, and open the C-terminal ring, could allow for prolonged ability to control the build up of hydrogen peroxide in the cellular milieu. This could be an important biological function since this is a mitochondrial enzyme; the respiratory chain is constantly producing hydrogen peroxide.

Base on the data presented herein, Figure 23 diagrams a proposed mechanism of TR. Of the two residues in the Cys-Cys or Cys-Sec dyad, the C-terminal residue is thought to be the nucleophile (X = S or Se) in the attack on the disulfide bond of Trx (Figure 23B). Once the mixed disulfide (or selenylsulfide) bond forms between enzyme and substrate (Figure 23C), the adjacent Cys residue must then act to resolve the complex and form the eight-membered ring. NMR data shows that substitution of a Se atom in the ring makes it larger and stabilizes the trans form of the ring. Thus for Sec-containing enzymes, this resolution step would be more facile. Once the eight-membered ring forms, it must be opened by an attacking sulfur nucleophile (CysIC) as shown in (Figure 23D). Our X-ray structure of DmTR shows that this must happen when the ring
is in a cis conformation (Figure 24). This cis conformation can be formed because the eight-membered ring can equilibrate between cis and trans ring forms. Thus I propose that a key difference between the two classes of enzymes is that Se-TRs can utilize the trans form of the ring because the Se atom does not need to be protonated. The presence of Se would also eliminate a trans to cis conformational switch that is needed in the Cys-TRs.

This report strongly argues in favor of our hypothesis, in which Sec incorporation into mammalian TR active site is essential for activity because of its superior ability to act as leaving group in the ring opening step of the reaction mechanism, not its enhanced nucleophilicity towards Trx.
Figure 22: Mechanism of truncated TR toward cyclic and acyclic peptide substrates. The peptide substrate is positioned in the active site such that nucleophilic CysIC thiolate can attack the Cys1 position, and cause the release of X (pink). A.) In the case of the cyclic peptide, when X = Cys, the ring is critical for positioning X near the conserved His, to receive a proton to stabilize the leaving group. When X = Sec, the position of the ring near the His is not as critical. B.) In the case of the acyclic peptide, when X = Cys, protonation of X is unlikely to occur due to the “floppy” nature of the peptide. However, when X = Sec, protonation does not need to occur for stabilization due to its lowered pKₐ.
Figure 23: Mechanism of Thioredoxin Reductase.

(A) Proposed pathway for transfer of electrons to Trx by TR. The Cys residue that interacts with the flavin cofactor is labeled as “CT” (charge-transfer), while the Cys residue that acts as the interchange residue is labeled “IC”. Once the interchange Cys becomes reduced by the flavin cofactor, it initiates attack on the eight-membered ring formed by either adjacent Cys residues (DmTR and CeTR2) or adjacent Cys and Sec residues (mTR3) on the C-terminus on the opposing subunit. I refer to this step in the reaction mechanism as the “ring opening” step and is highlighted in red in the diagram. Once this ring is reduced, the attacking nucleophile (either a sulfur atom or a selenium atom – labeled as X), initiates attack on the disulfide bond of Trx (B). The N-terminal Cys of the dyad would then “resolve” the mixed disulfide formed between TR and Trx and is thus labeled as “Res”. The “prime” designation indicates residues that are on the adjacent subunit. (C) Diagram of the TR/Trx complex formed when the attacking nucleophile (either S or Se) attacks the disulfide bond of Trx. The adjacent Cys residue then attacks to resolve this complex, releasing product and forming the oxidized, eight-membered ring. (D) The eight-membered ring is then reduced by CysIC. CysIC can attack the N-terminal sulfur atom (pathway 1) or the C-terminal atom (S or Se, pathway 2). I have argued here and previously (65, 88) for pathway 1). Term_{aa} is the C-terminal amino acid (either Gly or Ser).
The Cys-Cys eight-membered ring has been implicated in switching between trans (A) and cis (B) conformations. NMR data indicates that Se incorporation results in stabilization of the trans conformation limiting switching.

**Figure 24: Conformational Ring Switching**

The Cys-Cys eight-membered ring has been implicated in switching between trans (A) and cis (B) conformations. NMR data indicates that Se incorporation results in stabilization of the trans conformation limiting switching.
References


Appendixes

A. Abbreviations

$^{1}A_{280}$, absorbance at 280nm; $A_{412}$, absorbance at 412nm; $A_{460}$, absorbance at 460nm; AA₁-AA₂-AA₃-AA₄-Sec₁-Cys₂-AA₇-AA₈, peptide sequence for switch mutant; ADP, adenosine diphosphate; βME, β-mercaptoethanol; C. elegans, Caenorhabditis elegans; CeTR₂, mitochondrial TR from Caenorhabditis elegans; CeTR₂Δ₈, truncated mitochondrial TR from Caenorhabditis elegans; CeTR₂-GCCG, full-length mitochondrial TR from Caenorhabditis elegans indicative of tetrapeptide motif; CVNVGC, conserved N-terminal redox site with sequence Cys-Val-Asn-Val-Gly-Cys; Cys, cysteine; Cys₁, N-terminal cysteine of dyad; Cys₂, C-terminal cysteine of dyad; CysIC, interchange cysteine; CXXC, the tetrapeptide motif Cys-Xaa-Xaa-Cys; D. melanogaster, Drosophila melanogaster; DEAE, diethylaminoethyl; DmTR, TR from Drosophila melanogaster; DmTRΔ₈, truncated TR from Drosophila melanogaster; DmTR-SCCS, full-length TR from Drosophila melanogaster indicative of tetrapeptide motif; DmiTrx, cognate Trx from Drosophila melanogaster; DTNB, 5,5’dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; E. coli, Escherichia coli; EDTA, ethylaminediamine tetraacetic acid; Gly, glycine; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione, reduced; GSH I, interchange glutathione; GSH II, first leaving group glutathione; GSSG, glutathione, oxidized; H₂O₂, hydrogen peroxide; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; $k_{cat}$, turnover number; kDa, kilodaltons; $K_M$, Michaelis constant; LB, Luria-Bertani media; MDS, mixed disulfide; MES, 2-(4-morpholino)-ethane sulfonic
acid; MESNA, 2-(N-morpholino)ethanesulfonic acid sodium salt; MOPS, 3-(N-morpholino) propane sulfonic acid; Mr, molecular ratio; mTR3, mitochondrial TR from mouse; mTR3Δ8, truncated mitochondrial TR from mouse; mTR3-GCUG, full-length mitochondrial TR from mouse indicative of tetrapeptide motif; MW, molecular weight; MWCO, molecular weight cut off; NaCl, sodium chloride; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced; Ni-NTA, nickel nitrilotriacetic acid; NMA, N-methylmercaptoacetamide; O.D., optical density; PCR, polymerase chain reaction; PDB, Protein Data Bank; Pep-TNB, AA1-AA2-AA3-AA4-AA5-Cys1-S-S-TNB (5-thio-2-nitrobenzoate) mixed disulfide; PfTR, Plasmodium falciparum TR; PRTQGCCG, the octapeptide Pro-Arg-Thr-Gln-Gly-Cys-Gly; PTPASCCS, the octapeptide Pro-Thr-Pro-Ala-Ser-Cys-Cys-Ser; PTVTGCUG, the octapeptide Pro-Thr-Val-Thr-Gly-Cys-Sec-Gly; S, sulfur atom; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Se, selenium atom; Sec, selenocysteine; Sec1, N-terminal selenocysteine of dyad; Sec2, C-terminal selenocysteine of dyad; Ser, serine; TB, terrific broth; TE, Tris EDTA buffer; TNB-S-, 5-thio-2-nitrobenzoate anion; TR, thioredoxin reductase; Tris, tris-(hydroxymethyl)aminomethane; Trx, thioredoxin; U, the one letter code for selenocysteine; UGA, sense codon for translation of Sec; WT, wild type; X, represents either (S) sulfur or (Se) selenium atom.