

UVM ScholarWorks

Differentiating the Roles of Redox-Active Cysteine Residues in Plasmodium falciparum Thioredoxin Reductase by using a "Seleno Effect"

Item Type	undergraduate thesis
Authors	O'Keefe, John P
Download date	2026-05-09 14:39:47
Item License	http://creativecommons.org/licenses/by-nc-nd/3.0/
Link to Item	https://hdl.handle.net/20.500.14849/5690

Differentiating the Roles of Redox-Active Cysteine Residues in
Plasmodium falciparum Thioredoxin Reductase by using a
"Seleno Effect"

John O'Keefe¹

¹Department of Biochemistry, University of Vermont, College of Medicine, 89 Beaumont
Avenue, Given Building Room B413, Burlington, Vermont 05405, United States

ABSTRACT

The purpose of my research is to determine if the substitution of selenium for sulfur will cause rate acceleration of the thiol-disulfide exchange reaction in the C-terminal redox center of *Plasmodium falciparum* thioredoxin reductase (PfTR) in a position specific manner. *P. falciparum* is the protist that causes most serious form of malaria and is responsible for the majority of malaria related deaths. While mammalian thioredoxin reductase (mTR) contains selenium in its C-terminal redox center, which accelerates the rate of reaction, PfTR functions in the absence of selenium. The results of my studies show that the introduction of selenium via selenocysteine to the C-terminal redox center of PfTR impacts the rate of this reaction positively when substituted at Cys⁵⁴⁰ and negatively at Cys⁵³⁵. This data can be explained mechanistically by the use of a disulfide electrophilic activation mechanism by PfTR. Specifically, the disulfide bond between Cys⁵⁴⁰ and Cys⁵³⁵ in the C-terminal redox center of PfTR is primed for attack by the thiolate of Cys⁹³ of the N-terminal redox center because of a hydrogen bonding interaction between Cys⁵³⁵ and an adjacent histidine residue. This interaction polarizes the Cys⁵⁴⁰-Cys⁵³⁵ disulfide, making the sulfur atom of Cys⁵⁴⁰ highly electrophilic. The differential effects on rate caused by selenium substitution of each of the two sulfur atoms of the C-terminal disulfide represents a “seleno effect”, similar to the observed “thio effect” in phosphodiesterases when sulfur substitutes for oxygen in non-bridging oxygen atoms of phosphodiesteres. Thioredoxin reductase is essential in all organisms and its loss of function is deadly for the host. Due to the differences in structure between PfTR and mTR, the mechanism proposed represents a potential drug target in the fight against malaria.

The Thioredox System

The thioredoxin system is comprised of the small protein thioredoxin (Trx), thioredoxin reductase (TR), and NADPH. The components and function of this system is described in the following sections.

Thioredoxin.

Thioredoxin (Trx) is a class of small disulfide-containing oxidoreductases that is ubiquitous from archaea to humans.¹ The TRX gene encodes the human variant of the protein, and a loss of function mutation of this gene is lethal at the four cell stage of development.^{2, 3} Trx is a 12KD highly conserved protein and has multiple functions including 1) facilitating thiol-disulfide exchange between itself and a target protein 2) donating electrons to target proteins and 3) quenching reactive oxygen species (ROS) as an antioxidant.⁴ Its redox exchange capability is essential to many different processes throughout all cells and new systems are continually being discovered in which Trx plays a role. It has been found to be involved in regulation of metabolic pathways via redox signaling as well as through its control of oxidative stress.^{5, 6} The defining trait of Trx is the presence of two vicinal cysteine residues within its active site in a –Cys-Gly-Pro-Cys- amino acid motif. These cysteines exist as either the active, reduced form (Trx(SH)₂) or the inactive, oxidized, disulfide form (TrxS₂). When reduced Trx(SH)₂ reacts with a target protein it loses two hydrogens and becomes oxidized, subsequently reducing the target protein.⁷ Trx is kept in the reduced state by the flavoenzyme thioredoxin reductase, which then in turn utilizes NADPH to maintain *its* reduced state.⁴

Targets of Trx. One function of Trx is to reduce disulfide bonds on target proteins through thiol-disulfide exchange. This involves attack by one of the reduced sulfur groups within the Trx

active site on the target protein disulfide. This forms a mixed disulfide between Trx and its target sulfur, with the other sulfur that was previously part of the target disulfide becoming a thiolate. The remaining thiolate group on the Trx protein then attacks this mixed disulfide, forming a new disulfide between the two cysteine residues of Trx and releasing the newly reduced target protein, as is illustrated in **Figure 1**.⁸ This process of thiol-disulfide exchange is repeated when Trx reductase reduces the oxidized Trx, which then allows the Trx→target cycle to continue.

Targets of Trx that undergo this exchange include insulin, choriogonadotropins, coagulation factors, and glucocorticoid receptor, among many others.⁹ While the function of Trx in many cases is simply to keep intracellular protein disulfides reduced it can also serve as a regulatory protein.¹⁰ In plants it interacts with ferredoxin to regulate chloroplast photosynthetic enzymes, while in mammals it is used for the redox regulation of multiple transcription factors, including AP-1 and NFκB, to allow for communication between the nucleus and cytoplasm.^{11, 12} Through a different regulatory strategy reduced Trx can also interact with ASK1 in mammals to prevent downstream signaling of apoptosis.¹³

Trx also has a secondary function as hydride donor, which is analogous to the function of NADH or NADPH. It is used in conjunction with peroxidases called peroxiredoxins to turn ROS such as H₂O₂ into water or alcohols.¹⁴ Trx was originally purified from *Escherichia coli*, where it was isolated as a hydrogen donor for ribonucleotide reductase, an essential enzyme that converts ribonucleotides to deoxyribonucleotides.¹⁵ This interaction dictates that all organisms that have DNA must also have Trx, and partially accounts for the essential nature and ubiquity of Trx throughout evolutionary history. Other utilizations of Trx as a hydride donor include protein repair by methionine sulfoxide reduction and assimilation of sulfur by sulfate to sulfite reduction in bacteria and yeast.¹⁶

In addition to being a substrate for peroxiredoxins and transitively acting as an antioxidant through them, Trx can also quench ROS and act as a direct antioxidant. The reduced cysteine residues of the active site can donate electrons to ROS reducing them. The active site cysteines become oxidized to sulfenic acid or sulfinic acid as a result. This reaction is much slower than peroxiredoxin activity and as such its antioxidant capability is less. However, peroxidases can only act on hydrogen peroxide while Trx can quench superoxide and hydroxyl radical as well as hydrogen peroxide.¹⁷

Thioredoxin Reductase.

Thioredoxin reductases (TR) are members of the flavoprotein family of pyridine nucleotide disulfide oxidoreductases. Other enzymes in this class include lipoamide dehydrogenase, glutathione reductase, and NADH peroxidase. TR catalyzes the NADPH dependent reduction of Trx and other substrates by transferring electrons through a series of redox centers. All organisms express some form of TR and they are categorized into two major classes; a low-molecular weight (M_r) class of 35 KDa that includes bacteria, archaea, and some eukaryotes such as plants, and a high- M_r class of 55 KDa found in many animals, including all mammals. Both classes exist as homodimers, with each monomer possessing a prosthetic flavin (FAD) redox domain as well as one or two disulfide redox centers, depending on the class. The reaction to reduce thioredoxin or another substrate begins with NADH binding to the enzyme and reducing the flavin group to FADH₂. This initiates a chain of thiol-disulfide redox reactions within the enzyme, ending with the reduction of the substrate. Within the classes of TR enzymes there are subdivisions, and while all of them reduce Trx there are slight variations in the mechanisms which allow for some TR enzymes to have a wider range of substrates than others.

High Mr Thioredoxin Reductase. High M_r TRs contain a flavin prosthetic group redox center as well as two disulfide redox centers, one N-terminal and one C-terminal. The N-terminal redox center has a conserved sequence of -Cys-Val-Asn-Val-Gly-Cys- adjacent to the flavin domain. This motif is identical to the redox center of the related enzyme glutathione reductase, which uses the redox center to directly reduce the oxidized form of the glutathione tripeptide. In TR the N-terminal redox center reduces the C-terminal redox center through thiol-disulfide exchange, which then subsequently reduces Trx. This is illustrated in **Figure 2**. The C-terminal center varies between species and allows for further classification of the TR enzyme into selenocysteine (Sec) containing and cysteine (Cys) only redox centers. High M_r TR is highly divergent from low M_r TR, which does not contain a C-terminal redox center, and the sequence identity between the two was found to be only 31%, while the sequence identity between high M_r TR and glutathione reductase is 44%.¹⁸

Sec containing High Mr TR. In mammalian variants of the TR enzyme (mTR) the C-terminal redox center contains the rare amino acid selenocysteine (Sec). This redox active site has a tetrapeptide sequence of -Gly-Cys-Sec-Gly- and is conserved among all isozymes of mTR. In the amino acid selenocysteine, selenium replaces the sulfur atom of a cysteine residue and participates in redox reactions through thiol-disulfide like exchange. Mammalian TR enzymes with this C-terminal redox sequence are designated Type Ia. In humans there are three isozymes of mTR; cytosolic mTR, mitochondrial mTR, and testes-specific mTR. Cytosolic mTR is structurally unique in comparison to all other high M_r TR enzymes and within the literature it is categorized as TR1. Mitochondrial mTR is referred to as TR3 and most other high M_r TRs are referred to as TR3-like for their relative similarity to this variant. The reason for this distinction is that TR1 contains a “guiding bar” (amino acids 407-422) within its structure that prevents the

C-terminal redox center from moving away from the N-terminal redox center and exposing it.¹⁹ This is important because for enzymes that lack this guiding bar, such as TR3, the N-terminal redox center is exposed and can interact with substrates directly, bypassing the C-terminal redox center. This mechanism is analogous to the mechanism of substrate reduction by glutathione reductase. It has been shown that TR3 and TR3-like enzymes can reduce a wide range of substrates including 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), lipoic acid, and selenocystine without the presence of the active Sec residue in their C-terminal redox center by interacting with the N-terminal redox center directly. However, when a mutation is made to a TR1 enzyme to eliminate this Sec its catalytic activity is decreased over 100 fold for all of these substrates due its inability to apply the alternative reduction pathway through the N-terminal redox center.²⁰

Why Selenocysteine? Selenium is located directly below sulfur on the periodic table, and as such has many of the same chemical properties. However, because selenium is a much larger element with a bigger electron cloud it can both accept and donate electrons better than sulfur. While sulfur is a good electron donor, or nucleophile, it has less ability to accept electrons in comparison to selenium. Selenium can be described as a Janus-faced element, meaning that it can act as both as a very good nucleophile and a good electrophile, especially as part of a selenosulfide bond or as an *Se*-oxide. This allows selenium to perform chemistry in ways that sulfur cannot, and in the case of TR allows the selenium residue in the active site to attack one of the sulfurs of the Trx disulfide much faster and more reliably than a simple cysteine residue could. It has been shown previously that selenium not only acts as a nucleophile when it attacks the Trx disulfide, but it also acts as an electrophile during the thiol-disulfide exchange between the N-terminal and C-terminal redox centers.²⁰ This overall chemical advantage of selenium over sulfur is necessary in mTR to turn over Trx quickly and efficiently.²¹ Selenium also has a

chemical advantage over sulfur in its ability to resist inactivation by oxidation. When the sulfur atom in a cysteine molecule is exposed to ROS it can become permanently over-oxidized to the sulfinic acid form. This causes the cysteine residue loses its chemical reactivity and ability to perform redox reactions.²² However, the Janus faced nature of selenium prevents irreversible oxidation of selenocysteine, which is important because Trx is essential in eliminating ROS.²³ If TR became inactivated due to ROS it would not be able to turn over inactive Trx in the event of a sudden influx of ROS, which would defeat the antioxidant capabilities of the thioredoxin system.

Cysteine only enzymes. High Mr TR enzymes have been discovered in *Drosophila melanogaster* (DmTR) and *Plasmodium falciparum* (PfTR) that do not contain any selenocysteine in their respective amino acid sequences. They instead utilize two cysteine residues within their C-terminal redox centers. The structure of these redox centers differs between the two organisms, with *D. melanogaster* having a sequence of –Ser-Cys-Cys-Ser- and *P. falciparum* having one of –Gly-Cys-Gly-Gly-Gly-Lys-Cys-Gly. When oxidized the DmTR C-terminal redox center forms a ring of eight atoms, which is analogous to the structure of the oxidized C-terminal redox center of the mammalian variant of TR. DmTR is designated a Type Ib TR for this reason.²⁴

Alternately, the oxidized PfTR redox center forms a twenty membered ring, which among TR enzymes has only been discovered in apicomplexan TRs and as such is given its own category as a Type II TR.²⁵ Both of these are also classified as TR3-like enzymes as both their N-terminal and C-terminal redox centers can reduce substrates. *Drosophila melanogaster* actually lacks glutathione reductase so glutathione is a substrate for DmTR and is reduced within the N-terminal redox center, which is a reaction unique to insects.²⁶

Low Mr TR. While low Mr TRs and high Mr TRs are homologous, the two classes are structurally and mechanistically dissimilar. The *Escherichia coli* variant is the most widely studied low Mr TR so knowledge of the class is largely based on this specific enzyme. As mentioned previously, the low Mr TR class lacks the C-terminal redox center found in high Mr TRs and instead has a single disulfide redox center that directly reduces Trx via thiol-disulfide exchange. The mechanism of reduction begins with the binding of NADPH to its binding site. This induces a rotation within the enzymes structure to bring the NADPH adjacent to the flavin domain, which allows for rapid hydride transfer. At the same time the previously reduced nascent thiol redox center moves away from the flavin domain and rotates toward the surface of the protein to undergo thiol-disulfide exchange with the waiting Trx protein substrate. When NADP⁺ is released the reverse rotation brings the newly oxidized thiol redox center adjacent to the reduced flavin domain to be reduced. This process of rotation differs from the relatively static mechanism of the high M_r TR.²⁷

High Mr TR Mechanism.

The focus of this thesis is to solve the mechanics of electron flow between the N-terminal and C-terminal redox centers of PfTR, namely which cysteine of the C-terminal redox center undergoes attack by the N-terminal redox center and why this reaction is favorable. In order to solve this problem it is necessary to have an understanding of these mechanics within other high Mr TR variants. The sequence of the C-terminal redox center is a significant difference between the various types of high M_r TR enzymes and the reduction of the C-terminal center by the N-terminal center is the focus of my studies. The mechanism of this reduction step can be studied and determined through enzyme assays, which is the approach I have taken here.²⁸ Below is a description of the flow of electrons from NADPH to the substrate catalyzed by TR.

For all high Mr TRs the state of the enzyme immediately after reduction of a substrate exists as the flavin domain oxidized and hydrogen bonded to the N-terminal redox center, which is reduced. At this point the C-terminal redox center is an oxidized disulfide/selenylsulfide. The N-terminal center then undergoes thiol disulfide exchange with the C-terminal center, reducing it. NADPH then binds and the flavin is reduced to FADH₂. This immediately undergoes redox exchange with the N-terminal center, returning it to a reduced state. At this stage both redox centers are reduced. Trx(S₂) then binds to the C-terminal domain and undergoes thiol disulfide exchange. It leaves as Trx(SH)₂ and the TR enzyme is returned to its original oxidation state. It is important to note that the structure of TR is that of a homodimer, with the redox motifs of each monomer oriented as follows; C-terminal redox center, NADPH binding domain, flavin domain, and then N-terminal redox center. The two monomers are linked “head to tail” so that the N-terminal redox center of one monomer is reducing the C-terminal redox center of the *opposite* monomer. The transfer of electrons between these monomers is the process that this thesis is concerned with.

The mechanism of thiol/disulfide exchange of the C-terminal redox center of mTR. As previously stated, mTR enzymes have a C-terminal redox center sequence of –Gly-Cys⁴⁹⁵-Sec⁴⁹⁶-Gly-OH.²⁹ In the oxidized state (with a selenosulfide bond), this tetrapeptide forms an eight membered ring. This selenosulfide undergoes a thiol-disulfide exchange reaction with the N-terminal redox center of the opposite monomer of the homodimer. The N-terminal center is composed of an interchange Cys residue (Cys_{IC}) and a charge transfer Cys residue (Cys_{CT}) that form a disulfide bond in the oxidized state. This N-terminal disulfide is reduced by the flavin group. This reduction results in a nucleophilic Cys_{IC} that attacks the electrophilic Sec residue of the vicinal selenosulfide bond and forms a new mixed selenosulfide bond connecting the two redox centers.

The thiolate of the Cys_{CT} then attacks Cys_{IC}, reforming the oxidized N-terminal disulfide bond and leaving the C-terminal redox center fully reduced as a thiol and a selenolate. This Sec residue then attacks the vicinal disulfide of Trx(S₂), acting this time as the nucleophile in the thiol-disulfide-like exchange. This leaves the C-terminal center oxidized and Trx reduced.²⁸ The utilization of the unique chemical capabilities of selenium in this mechanism allow for rapid intramolecular electron exchange and resistance to inactivation by oxidation.

DmTR C-terminal Redox Mechanism. The C-terminal redox center of DmTR is a tetrapeptide with an active site sequence of –Ser-Cys⁴⁸⁹-Cys⁴⁹⁰-Ser-COO⁻. When in the oxidized disulfide state this motif forms an eight-membered ring similar to the one formed in this complex within mTR. However, because this redox center lacks Sec it must somehow mimic the properties of selenium within one of the Cys residues to allow rapid electron transfer to occur. The enzyme does this by positioning a positively charged histidine (HisH⁺) residue adjacent to the Cys⁴⁸⁹ of the disulfide. The positive charge of the His induces a negative charge on the Cys⁴⁸⁹ sulfur, which creates a dipole within the disulfide and gives the sulfur of Cys⁴⁹⁰ a partial positive charge. By doing this the Cys⁴⁹⁰ is made electrophilic and can undergo attack by the Cys_{IC} residue of the N-terminal redox center. In the literature this mechanism has been deemed “electrophilic activation”.³⁰ After the C-terminal redox center has been reduced it can undergo thiol-disulfide exchange with Trx(S₂) by attacking the disulfide bond of Trx(S₂) with Cys⁴⁹⁰. This reaction is made favorable by the presence a Ser residue adjacent to Cys⁴⁹⁰ that stabilizes its sulfur as a thiolate through hydrogen bonding after reduction. A thiolate is a much better nucleophile than the thiol group that would form in the absence of the Ser residue and allows the C-terminal redox center to reduce the substrate at a more rapid rate.²⁹ Through these mechanisms Cys⁴⁹⁰ is made chemically similar to Sec during the redox reactions involving the C-terminal

center, allowing the enzyme to perform the same chemistry as mTR without the need for selenium.

PfTR Proposed Mechanism. The C-terminal redox center of PfTR is structurally very different from the other two variants discussed, but it is hypothesized in this thesis that the redox mechanisms are similar. The active site sequence of this enzyme is –Gly-Cys⁵³⁴-Gly-Gly-Gly-Lys-Cys⁵⁴¹-Gly-COO⁻, which forms a twenty-membered ring in the oxidized state. The presence of the disulfide introduces this motif to undergo a transition from an intrinsically disordered state to a β -turn- β motif, which is stabilized by hydrogen bonding between the amino acids of the ring. Deletion or substitution of any of the amino acids of the sequence decreases the catalytic activity of the enzyme.²⁵ The unique structure of this C-terminal redox center allows this enzyme to specifically reduce its native substrate, the *Plasmodium falciparum* variant of Trx (PfTrx).³¹ Although this ring is much larger than those of DmTR and mTR, I propose that it undergoes electrophilic activation in a way similar to the mechanism of DmTR. Like DmTR, a positively charged histidine residue (His⁵⁰⁹) is located adjacent to the C-terminal redox center of PfTR. I hypothesize that this residue hydrogen bonds with the more N-terminal cysteine residue of the redox center disulfide, Cys⁵³⁵, to induce a positive charge on more C-terminal cysteine, Cys⁵⁴⁰, priming it to be attacked by the N-terminal redox center. This is illustrated in **Figure 7**. These assigned roles for the cysteine residues are based on the mechanisms of mTR and DmTR.^{28, 32}

Inhibition of Hydrogen Bonding

Thio effect. The substitution of sulfur in place of phosphate bound oxygen is a tool mainly used to determine the mechanisms of RNA phosphodiester bond cleavage by ribozymes and DNA phosphodiester cleavage by phosphodiesterases like RNase A.^{33, 34} As sulfur is directly below

oxygen on the periodic table it less electronegative, larger, more readily polarizable, and has a lower pK_a . When oxygen is replaced with sulfur within a phosphate group many properties of that group can be altered, including its hydrogen bonding capabilities, the geometry of the phosphorane intermediate, its leaving group property, and its affinity to metal ions.³⁵ The substitution of sulfur at the different oxygen positions within a phosphate group will typically decrease the K_{cat} of the enzyme being studied and allow for insights into the mechanism of catalysis. This change is expressed as a ratio of k_O/k_S , where O is the native oxygen substrate and S is the sulfur analog, and this ratio is called the “thio effect”.³⁶ A common example of this is the substitution of sulfur for oxygen at a non-bridging oxygen atom of a phosphate group. Typically the phosphorous atom is activated for nucleophilic attack from an enzyme by hydrogen bonding between the non-bridging oxygen and a positively charged hydrogen bond donor. The lower basicity of sulfur in comparison to oxygen results in a lower ability of the sulfur atom to engage in hydrogen bonding or to formally accept a proton. This results in less activation of the phosphorous atom of a phosphorothioate compared to a phosphorous atom of a phosphate, resulting in a slower rate of reaction.³⁷

Seleno Effect. This concept can be extended to the replacement of a native sulfur atom in a disulfide bond with the element below it on the periodic table, selenium. The differences between Se and S can be used to explore mechanisms in the same way that O to S substitution can. This concept has been used to account for differences in hydrogen bonding between Cys and Sec residues within the active sites of enzymes due to the lower pK_a of Sec compared to Cys. Within the context of TR, the Cys residue of the C-terminal redox center that is not attacked during redox exchange undergoes hydrogen bonding with an adjacent histidine residue, which in the case of DmTR and likely PfTR induces a dipole in the disulfide and allows the Cys to

become protonated easily upon reduction. This same mechanism was proposed for the PfTR mechanism.²⁵ When this activating C-terminal Cys in DmTR was replaced with Sec the rate of the thiol disulfide exchange reaction was decreased by 10-fold.³⁰ The substitution of Se in place of S in the redox center disulfide lowered the pKa of the residue and in doing so decreased the effect of hydrogen bonding by an adjacent positively charged histidine residue, and resulted in a less polarized selenosulfide bond. This made the disulfide less likely to undergo attack by the N-terminal redox center and lowered the overall rate of thiol disulfide exchange. It is hypothesized that a similar substitution of the activating Cys→Sec in PfTR will result in a comparable decrease in activity due to this seleno effect. The mechanism by which this occurs is shown in **Figure 8**.

The purpose of this project is to determine the mechanism of redox exchange between the N-terminal and C-terminal redox centers of PfTR. In order to isolate which cysteine residue of the C-terminal redox center is being attacked, as well as identify if hydrogen bonding is occurring to polarize the disulfide, the seleno effect was utilized through substitution of the cysteine residues with selenocysteine. It is hypothesized that an electrophilic activation mechanism exists in the C-terminal redox center of PfTR through hydrogen bonding between His⁵⁰⁹ and Cys⁵³⁵, which induces a partial positive charge on Cys⁵⁴⁰ and makes it more prone to attack by the N-terminal redox center.

MATERIALS AND METHODS

Materials. Vent DNA polymerase, NdeI, SapI, T4 DNA ligase, E. coli ER2566 competent cells, and chitin-agarose beads were purchased from New England Biolabs (Ipswich, MA). GSH - sepharose 4 Fast Flow, benzamidine sepharose, and 2'-4'-ADP-sepharose resins were obtained from GE Healthcare (Waukesha, WI). Solvents for peptide synthesis were purchased from EMD Biosciences (San Diego, CA). Fmoc-amino acids were purchased from Synpep Corp (Dublin, CA). Fmoc-Sec-Xanthene was obtained as a gift from Dr. Steven Flemer³⁸. Resins for solid-phase synthesis were purchased from Applied Biosystems (Foster City, CA). All other chemicals were purchased from either Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA). The HPLC system was from Shimadzu with a Symmetry® C₁₈-5 µm column from Waters (4.6 x 150mm). A Voyager-DE™ PRO Workstation (Applied Biosystems) was used for mass spectral analysis of peptide samples. All enzyme kinetic assays were performed on a Cary50 UV-vis spectrophotometer (Cary, Walnut Creek, CA) and were conducted at room temperature.

Production and Purification of Semisynthetic PfTR mutants and PfTRΔ7. The production of enzymes A and B of **Table 2**, as well as the PfTRΔ7 that was used in the peptide assays, was achieved through intein-mediated peptide ligation³⁹. First a truncated PfTR construct, PfTRΔ7, was formed by through PCR amplification of the full length wild type PfTR DNA template. The upstream primer that introduced a NdeI restriction site (sequence: 5'-ACAGACATATGGGATCCTGC-AAA-3') and a downstream primer which introduced a seven amino acid deletion, the insertion of a Cys residue, and a SapI endonuclease restriction site (sequence: 5'-ACAGCCGCTCTTCAGCAACCACCTTT-3') were used. PCR was done on an Eppendorf Mastercycler using Vent DNA polymerase. The amplified sequence was then digested with NdeI and SapI and shuttled into the pTYB1 expression vector from New England Biolabs.

Ligation of the product was then conducted using T4 DNA ligase. The resulting plasmid was used to produce the PfTR Δ 7-intein-chitin binding domain fusion protein in *E. coli* ER2566 cells with *Sce* VAM intein (both provided by New England Biolabs).

These cells were grown in Terrific broth medium with niacinamide, riboflavin, and pyridoxine supplementation (20 mg/L each). The cell culture, containing 0.2 μ g/mL ampicillin, was grown at 37 °C until the OD₆₀₀ reached ~0.6. The cells were then cooled to 20 °C, and IPTG was added to induce expression to a final concentration of 0.5 mM, with subsequent incubation done at 20 °C overnight on a shaker. The cells were harvested by centrifugation (9000 rpm in a Beckman J2-21 preparatory centrifuge for 10 min at 4 °C) following overnight incubation. The harvested bacterial cell pellet was normalized in chitin buffer A [50 mM MOPS and 150 mM NaCl (pH 7.0)] followed by cell lysis via pulse sonication. The lysed slurry was then pelleted via centrifugation (12000 rpm in a Beckman J2-21 preparatory centrifuge for 1 h at 4 °C), and the cleared lysate was loaded onto a chitin–agarose column pre-equilibrated with chitin buffer A. The intein-chitin binding domain fused to PfTR allowed it to bind to the resin with high specificity. The loaded PfTR protein was further washed with 0.5 L of chitin buffer A followed by additional washing with 0.5 L of high-salt chitin buffer B [50 mM MOPS and 500 mM NaCl (pH 7.0)]. Chitin buffer B was then added to the resin containing the bound protein to convert it to slurry, which was then transferred to two 50 mL conical tubes.

The semisynthetic PfTR enzymes (A and B of **Table 2**) were produced via ligation of desired synthetic peptides to the truncated PfTR Δ 7 containing a C-terminal thioester reactive group. Proteins containing a C-terminal thioester can be ligated to peptides that have an N-terminal Cys residue to form an amide bond³⁹. Simultaneous cleavage of the fusion protein and ligation with synthetic peptide were achieved by addition of a cleavage cocktail consisting of 120

mM N-methyl mercaptoacetamide (NMA) and the desired synthetic peptide in chitin buffer B (pH 8.0–8.5). The cleavage and ligation reaction was done at room temperature overnight with constant shaking. Following overnight incubation, the resin/chitin slurry was poured back onto the column and the liberated semisynthetic enzyme was eluted via gravity flow and washing with chitin buffer B. The semisynthetic enzyme was concentrated and buffer exchanged with buffer C [10mM NaCl, 10mMTris-EDTA, and 20 mM β ME (pH 8.0)] via ultrafiltration (Amicon Ultracel 30 kDa cut off filters). It was then loaded by gravity onto a pre-equilibrated 2',4'-ADP-sepharose column (20 mL). After the sample had been extensively washed with buffer C, the semisynthetic PfTR enzyme was eluted off the 2',4'-ADP-sepharose column with high-salt buffer [10 mM Tris-EDTA, 1 M NaCl, and 20 mM β ME (pH 8.0)]. The fractions containing PfTR were confirmed by spectrophotometry and SDS–PAGE, pooled, and then buffer exchanged via ultrafiltration with storage buffer [50 mM potassium phosphate, 500 mM NaCl, and 1 mM EDTA (pH 8.0)]. The concentration of each PfTR semisynthetic enzyme was determined through spectral analysis via the absorbance maximum of the flavin at 460 nm ($\epsilon = 22.6 \text{ mM}^{-1} \text{ cm}^{-1}$, representing the dimer).

The truncated enzyme (PfTR Δ 7) was produced for use in kinetic peptide substrate assays by cleaving the PfTR Δ 7–intein fusion protein in the presence of NMA, without peptide present. After cleavage from the chitin–agarose column, pooled fractions of PfTR Δ 7 were concentrated by ultrafiltration and loaded onto a Sephacryl-S200 column that had been pre-equilibrated with 50mM potassium phosphate, 500 mM NaCl, and 1 mM EDTA (pH 8.0). TR-positive fractions eluted off the column and were then pooled and concentrated using the methods above.

Syntheses of Cysteine Peptides. All peptides were synthesized with a Burrell Model 75 wrist-action shaker (Pittsburgh, PA). Peptides were synthesized on a 0.3 mmole scale using 2-

chlorotridylchloride resin (1.33 mmol/g loading) at 3 eq. excess. For each peptide synthesis the resin was first swelled in dichloromethane (DCM) for 30 min. and then the first amino acid was coupled using 1.1 eq. Fmoc-AA-OH dissolved in 5 mL of a 2.22% N-methylmorpholine (NMM)/ 97.7% DCM (0.2 M NMM) solution and shaken for one hour. The resin was then washed four times using DCM and capped with 10 mL of 8:1:1-DCM:Methanol:NMM and shaken for fifteen minutes. Following this the resin was washed 4X with DCM and 4X with dimethylformamide (DMF). Deprotection of the N-Fmoc protecting group was carried out using two 10-min agitations with a solution of 20% piperidine/ 80% DMF. Success of all deprotections and amino acid couplings were monitored qualitatively using a ninhydrin test⁴⁰. Elongation of the peptides used standard Fmoc SPPS chemistry with 2 eq. (equivalents relative to peptide synthesis scale in mmoles) HATU and 2 eq. Fmoc-AA-OH in 5 mL of a 4.40% NMM/ 95.6% DMF (0.5 M NMM) solution, shaken for one hour⁴¹. Following a final deprotection and 4X wash with DCM these peptides were cleaved from the resin with a cocktail consisting of 96:2:2 trifluoroacetic acid(TFA):triisopropylsilane(TIS):H₂O for 2 hr. The solution was blown into a conical using nitrogen gas and the resin was washed four times using DCM. The cleavage cocktails were dripped into cold, anhydrous diethyl ether to precipitate the peptide and then pelleted by centrifugation. The pellet was allowed to dry and then redissolved in 1 mL of the 96:2:2 TFA:TIS:H₂O cocktail, dripped into anhydrous diethyl ether and centrifuged to pellet (2X). The peptide was then dissolved in a minimal amount of a water/acetonitrile mixture and lyophilized. This process is illustrated in **Figure 3**.

Syntheses of Selenocysteine Peptides. The amino acid Fmoc-Sec-xanthene was used as the selenocysteine residue for the two Sec peptides. The resin and procedure for these peptides was the same as described previously, with one exception. When coupling Fmoc-Sec-Xan and all

other amino acids following it in the synthesis the conditions were 2 eq. Fmoc-AA-OH and 4 eq. HOAT dissolved in 5 mL of 1.88% diisopropylcarbodiimide (DIC)/98.1% DMF (94 μ L/4.906 mL) shaken for three hours for Sec or one hour for any other amino acid. The cleavage and swelling of the resin used the same procedure as for the cysteine amino acids.

Oxidation of Wild Type Peptide. During the synthesis of the wild type peptide Fmoc-Cys-S-Tmp was used as the cysteine building block in both locations along the peptide. After the final coupling but before the final deprotection and cleavage from the resin this peptide was oxidized. To do this 0.1 M NMM (55 μ L) in 5% dithiothreitol (DTT)/ 95% DMF was first added to the resin and shaken for 5 min. (3X) to deprotect the STMP group⁴². The resin was then washed 4X with DMF and 4X with DCM. The success of the deprotection was tested using a solution of Ellman's reagent and DCM on a sample of the resin beads. The resin was then washed 4X with DMF. 2 eq. of N-chlorosuccinimide dissolved in 5 mL of DMF was then added to the resin and shaken for 15 min. to form the disulfide bond. After washing 4X with DMF and then DCM the resin was re-tested to confirm the oxidation. The final Fmoc group was then deprotected and the peptide was cleaved from the resin. This process is illustrated in **Figure 4**.

Oxidation of the Homocysteine Peptides. Unlike the other peptides synthesized, the two homocysteine containing peptides were oxidized post cleavage from the resin. CLEAR-OXTM resin was used to induce disulfide formation of the disulfide bond. This resin has 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB/ Ellman's reagent) bound to a solid resin, was used to form the disulfide bond. The CLEAR-OXTM resin was loaded at a 0.21 meq./g of resin ratio compared to the peptide quantity, swelled in DCM for 40 min, then washed with DCM, DMF, methanol, deionized water, and degassed 1:1 100 mM ammonium bicarbonate:acetonitrile 3 times each.. The solid peptides (2 and 3) were then dissolved in 1 mL/6 mg of 1:1 acetonitrile (ACN): 0.1 M

ammonium bicarbonate (degassed). The solution was added to the resin and agitated for two hours. Following agitation by shaking the solution was blown out of the vessel with N₂ gas, diluted with water, and lyophilized. It was subsequently discovered that some DNTB and TNB anion had detached from the resin and contaminated the sample. These impurities were removed by binding the sample to DEAE-Sepharose resin (diethylaminoethanol). The impurities are negatively charged at this pH, while the peptide is positively charged and unable to bind the resin. The sample was dissolved in ammonia bicarbonate buffer, pH 7.5, and then added to a small amount of resin that had been equilibrated with the same buffer. After agitating the sample with the resin for 30 min, the resin slurry was transferred to a microcentrifuge tube fitted with a filter. The eluent with the peptide was then collected by centrifugation and lyophilized. The oxidation process is shown in **Figure 5**.

Oxidation of Selenocysteine Peptides. The oxidation of the Sec peptides occurs during their cleavage from the resin. The xanthene protecting group was cleaved by the highly acidic TFA:TIS:H₂O cleavage cocktail. The free oxygen in solution then allows the highly reactive selenium to spontaneously oxidize with the cysteine residue forming the seleno-thiol bond. This is illustrated in **Figure 6**.

Confirmation of Peptide Sequences. The purity, sequence, and oxidation of all synthesized peptides were confirmed through mass spectral analysis using a Voyager-DETM PRO Workstation (Applied Biosystems) based on calculated molecular weights for the peptides. These are shown in **Figures 15, 16, 17, 18, and 19**.

Enzyme Assays. All assays were done using a spectrophotometer at A₃₄₀. The parameters for all assays were 500 μ L total volume, 1 mM EDTA, 150 μ M NADH, 100 mM KPO₄ buffer, 15 nM

PfTR Δ 7 truncated enzyme, and varying synthesized peptide concentrations. The assays measured the consumption of NADH at A_{340} , which was then used to calculate enzyme-peptide activity using Beer's Law.

Failed Oxidation of Peptides. Multiple copies of all synthesized peptides were made, but due to the nature of the enzyme assay not all could be used due to the oxidative reagents utilized to cyclize the disulfide or selenosulfide bond. Initially peptides I, II, and III of **Table 1** were oxidized using a molar equivalent of benzeneseleninic acid. This reagent was later determined to give artificial activity to the enzyme assays. An extraction was attempted using 1:1 Phenol : Chloroform, but not all of the contaminant was removed and some peptide was lost. Another oxidation was done on peptide I only, after resynthesizing it, using DTNB in solution. This also resulted in contamination and artificial activity, and again extraction using phenol-chloroform was unsuccessful. Oxidation was then attempted again on resynthesized peptides II and III using CLEAR-OX™ resin. This was also found to contaminate the samples with DTNB, so extraction was done with DEAE-Sepharose resin as described above, and was successful. The alternate Fmoc-Cys-S-Tmp synthesis described above was then used to oxidize peptide I on-resin, and was successful.

RESULTS AND DISCUSSION

Electrophilic Activation: Isolation of N-terminal to C-terminal thiol disulfide exchange

In nature Cys orthologs of Sec enzymes have been shown to possess mechanisms to increase S-nucleophilicity that allow for increased rates of thiol disulfide exchange, to compensate for the absence of the more nucleophilic selenium.²⁹ Selenium is also a more electrophilic atom than sulfur, and so it stands to reason that Cys orthologs of Sec enzymes in which selenium acts as an electrophile have another mechanism to emulate this superior electrophilicity.⁴³ In mTR and other Sec TR enzymes selenium in the C-terminal redox center acts as both an electrophile during N-terminal to C-terminal redox exchange and a nucleophile during its attack on a substrate.^{28, 44} In order to catalyze the same reaction, TR enzymes that lack Sec, such as DmTR and PfTR, must have mechanisms that change the properties of sulfur at these steps to emulate the chemistry of selenium. Previous work investigating N-terminal to C-terminal disulfide exchange in DmTR provides evidence that the disulfide bond of the C-terminal redox center is polarized by an adjacent histidine residue, inducing a partial positive charge on one of the sulfurs and priming it for attack by the N-terminal redox center. This mechanism was termed “electrophilic activation”.³⁰ Although the structures of the C-terminal redox center differs between DmTR and PfTR I hypothesize that the two enzymes use a similar mechanism of electrophilic activation to compensate for the absence of selenium in the thiol/disulfide exchange step that occurs between the N- and C-terminal redox centers, as illustrated in **Figure 7**.

To investigate this intramolecular mechanism I exploited the structure of PfTR to create an intermolecular reaction instead. The C-terminal redox center of PfTR is conveniently located

as the final eight amino acids of the enzyme's sequence. It is also present on the exterior of the protein, as it needs to interact with substrates. This allowed me to chemically synthesize peptides, either of the wild type sequence or mutants, and assay them in a solution containing a truncated variant of the enzyme (PfTR Δ 7) which does not contain the C-terminal redox center. The peptide binds to PfTR Δ 7 as a substrate and is reduced by the N-terminal redox center as it would be in the native enzyme, consuming NADPH in the process, as shown in **Figure 10**. By creating mutant peptides that have had a cysteine replaced with either selenocysteine or homocysteine I was then able to investigate the mechanism of this thiol disulfide exchange step. The synthesized peptides are illustrated below in **Table 1**. This method of assaying a truncated enzyme with a peptide as a substrate has also been used to investigate the C-terminal redox center mechanism of DmTR and mTR.^{21, 30}

To provide an alternate method of analyzing the exchange mechanism, semisynthetic enzymes of PfTR were also created. These were made by synthesizing mutant peptides with the same sequences as peptides IV and V (sans the N-terminal glycine residue) and ligating them to the truncated PfTR Δ 7 enzyme using methods previously published by the Hondal lab.²⁵ This allowed me to determine the activity of these mutant redox centers on two natural substrates of PfTR; Trx and selenocysteine. The results of these assays are shown in **Table 2** and **Figures 13** and **14**.

Assessing the impact of Cys⁵⁴⁰ \rightarrow Sec substitution

In mTR and other selenium containing TRs Sec is located at the more C-terminal position within the redox center, and in DmTR the most C-terminal cysteine residue has been shown to perform the same function that Sec does in mTR.⁴⁵ Based on this it is hypothesized that the most

C-terminal cysteine residue of the C-terminal redox center of PfTR, Cys⁵⁴⁰, takes on the role that Sec plays in mTR. If this hypothesis is correct, then the sulfur atom of Cys⁵⁴⁰ is attacked during thiol/disulfide exchange and then subsequently initiates nucleophilic attack onto the disulfide bond of the substrate. To test this hypothesis, a mutant peptide (peptide IV in **Table 1**) with a Cys⁵⁴⁰→Sec substitution was synthesized. When assayed this peptide resulted in a significant increase in PfTR Δ 7 activity when compared to the WT (peptide I), as illustrated in **Figure 11**. The effect of this substitution was also analyzed in a full length PfTR enzyme with its natural substrates through semisynthesis. The semisynthetic analog of this mutant peptide, ligated enzyme A, had Trx and selenocystine activity ~75 and ~25 fold higher than ligated enzyme B, which has Cys⁵³⁵→Sec substitution. The reason for this change in catalytic function is that by replacing sulfur with the more electrophilic selenium the N-terminal redox center has a better target for thiol disulfide exchange and the rate is consequently increased, as shown in **Figure 9**. The rate increase at this substitution position provides evidence that Cys⁵⁴⁰ is indeed the residue that is attacked during the intramolecular N-terminal to C-terminal redox exchange.

The question that then presents itself is that if Sec in this position does increase catalytic function, why is it not included in the wild type enzyme? There are four selenoproteins coded for by *P. falciparum*, so the organism has the correct “machinery” available to incorporate this amino acid into proteins.⁴⁶ A possible reason is that when a Cys⁵⁴⁰→Sec substitution is introduced the hydrogen peroxidase activity of PfTR goes up 6-fold, likely due to selenium’s ability to not be irreversibly inactivated by oxidation as sulfur is.^{23, 25, 47} Its increased ability to process hydrogen peroxidase could alter the redox balance of the cell and negatively affect some processes of the organism. Another possible reason is that PfTR is specialized to reduce PfTrx, hence its unique C-terminal structure. Although it lacks Sec, PfTR has similar activity reducing

PfTrx to human TR reducing human Trx, while having very little activity on human Trx or those of other species.⁴⁸ The specialization of PfTR's C-terminal redox center is important for differentiation between PfTrx, its host's human Trx, and its vector's *A. gambiae* Trx, so if Sec was introduced into this site it could negatively affect the enzyme's ability to differentiate. The PfTR C-terminal redox center could possibly be exploited as a potential anti-malarial drug target due to this unique specialization, but further research would need to be done.

Assessing Cys⁵³⁵ → Sec substitution: The Seleno Effect

While the results of peptide 4 did confirm that Cys⁵⁴⁰ is the residue undergoing attack by the N-terminal redox center, it did nothing to prove the electrophilic activation hypothesis. To test this hypothesis I synthesized peptide V, which has a Cys⁵³⁵ → Sec substitution. If we assume that it is Cys⁵⁴⁰ that is undergoing attack, then the electrophilic activation hypothesis predicts that it must be Cys⁵³⁵ that is interacting with an adjacent positive charge and polarizing the disulfide. If this mechanism was not present in the enzyme then conventional chemical logic would assume that a Cys⁵³⁵ → Sec substitution would result in an increase in catalytic activity, as selenium is a better leaving group than sulfur due to its lower pKa.²¹ As illustrated in **Figure 11** and **Table 1** this is not the case, with peptide V actually having a ~5-fold decrease in activity compared to the wild type peptide. This leads to the assumption that not only is there an electrophilic activation mechanism present in the wild type peptide that increases its activity, but also that selenium substitution for sulfur inhibits this mechanism in some way. In the semisynthetic enzyme assays ligated enzyme B had significantly lower activity than ligated enzyme A, which provides further evidence for this inhibition. I hypothesize that this inhibition is due to a “seleno-effect” on the hydrogen bonding between Cys⁵³⁵ and His⁵⁰⁹. Due to the differential effects seen in the two types of selenium substitution, the data is consistent with an electrophilic activation mechanism in the

reduction of the wild type peptide that increases its activity compared to not being activated due to polarization of the disulfide. In the semisynthetic enzyme assays ligated enzyme B had significantly lower activity than ligated enzyme A, which provides further evidence for the proposed electrophilic activation mechanism.

The term “seleno-effect” is a derivative of the thio-effect, a phenomenon commonly utilized in studying phosphodiesterases. In the thio-effect the substitution of sulfur in place of a non-bridging oxygen within the DNA phosphate backbone disturbs hydrogen bonding between a binding protein and the phosphate group and subsequently results in a measurable decrease in activity.³⁵ This disruption of hydrogen bonding is due to the lower pK_a of sulfur compared to oxygen, resulting in a less stable bond to a positively charged residue on the enzyme.³⁴

The periodic column of chalcogens includes, in descending order, oxygen, sulfur, and selenium (as well as tellurium and polonium which are not present in organisms). These elements share common qualities, such as having six valence electrons, which leads to them having a similar role within organic molecules. However, many qualities of these atoms change as you go down the column, including increased size, decreased electronegativity, and decreased pK_a s. The seleno-effect employs this change in basicity in the same way that the thio-effect utilizes the difference in basicity between oxygen and sulfur, by disturbing a hydrogen bond that would otherwise serve a role within the enzyme.

In the case of PfTR, it is the hydrogen bond that between Cys⁵³⁵ and His⁵⁰⁹ that will be disturbed. By replacing Cys⁵³⁵ with Sec in peptide 5 the lower basicity of selenium will not allow it to hydrogen bond as strongly to His⁵⁰⁹ as sulfur would. Following the electrophilic activation hypothesis, it is this hydrogen bond that is polarizing the disulfide in the wild type and inducing

a partial positive charge on Cys⁵⁴⁰, making it more electrophilic and prone to thiolate attack. So by eliminating this bond the selenosulfide bond does not become polarized and Cys⁵⁴⁰ incurs no positive charge, decreasing the rate of attack by the N-terminal redox center. This is shown in

Figure 8.

Assessing the importance of the C-terminal disulfide position

One of the features of the PfTR C-terminal redox center that I explored was the importance of the position of the active disulfide in relation to the rest of the enzyme. To do this I synthesized mutant peptides (II and III) with a homocysteine residue substituted for cysteine and assayed them with the truncated enzyme (PfTR Δ 7) to assess their activity. Homocysteine substitution, which when compared to cysteine has an additional methylene unit (3 Å) between the α -carbon and the thiol group, results in a peptide that forms a 21-membered ring when oxidized. This seemingly minute change to the native 20-membered ring results in 3-fold loss of activity when compared to the WT- peptide (I), as shown in **Table 1** and **Figure 12**. Previous research has shown that cysteine \rightarrow homocysteine substitution in the C-terminal redox center of semisynthetic PfTR enzymes results in a significant decrease in activity, and my research supports these findings through the alternate method of peptide substrate assays.²⁵ There are two possible mechanisms for this decreased activity 1) this change in disulfide positioning affects the rate of attack by the C-terminal thiolate on Trx or 2) the mutant peptide is an inferior substrate for N-terminal attack and thiol disulfide exchange. As the N-terminal \rightarrow C-terminal redox exchange is the rate limiting step of the enzyme the latter seems to be a more likely option. This is consistent with the proposed electrophilic activation of the disulfide by His⁵⁰⁹. By substituting homocysteine for cysteine the disulfide has a slightly altered location in the mutant peptide relative to this His⁵⁰⁹ residue, which disturbs the hydrogen bonding between His⁵⁰⁹ and Cys⁵³⁵,

thereby inhibiting electrophilic activation. This disruption of His⁵⁰⁹-Cys⁵³⁵ hydrogen bonding will occur regardless of which cysteine residue is substituted with homocysteine, as the shift in disulfide location will be roughly equal for substitution at either location. The results of the assays reflect this, with very little difference in activity between the two homocysteine mutant peptides (II and III).

Failed oxidations of peptides

The most difficult part of this research was not any of the processes involving assays or peptide synthesis, but rather the oxidation of the synthesized peptides. In order to prepare the peptides for assays they must be in the cyclized, oxidized form with either a disulfide or a selenosulfide bond between the second and seventh residues. There are many common reagents for this process, but the problem is that many of these same reagents are targets for TR. Even when using PfTR Δ 7 they can be reduced by the N-terminal redox center and give artificial activity to the assay. Luckily, the Sec containing peptides (IV and V) will auto-oxidize after the protecting group has been removed from the selenium residue, so there was no difficulty oxidizing those two peptides. The first oxidizing reagent that I used was benzeneseleninic acid to oxidize peptides I, II, and III. After we realized that the activities for all of these assays were far too high we concluded that the oxidizing agent was contaminating the peptides and causing artificial activity. Extraction of the benzeneseleninic acid from the peptides with phenol-chloroform did not manage to completely remove the contaminant so I discarded those samples and moved on. The next reagent that I tried was DTNB on peptide I only, which had the same result as the diphenyl diselenide in that it gave artificial activity and was very difficult to extract. This time I opted to extract the DTNB with HPLC, which did yield some fractions of pure peptide sample. However, these fractions did not contain enough peptide to perform full assays

and the sample was again discarded. After the DTNB failure I decided to oxidize my new samples of peptides II and III with Clear-OxTM resin, which has DTNB bound to a solid resin and hypothetically would allow me to remove the peptide after oxidation without any contamination. This did not prove to be the case as the peptide solution was yellow after removal from the resin, which is a telltale sign of TNB anion contamination. An extraction using HPLC was then attempted, but as with the previous sample, the fractions containing the peptide were very dilute and did not yield enough peptide after lyophilization to run a full assay. I then synthesized a peptide I that would allow for on-resin oxidation, so that any oxidizing agents used could simply be washed off of the peptide resin prior to cleavage. By utilizing Fmoc-Cys-S-Tmp instead of the acid labile Fmoc-Cys-(Trt)OH I was able to remove the S-Tmp protecting group with the mild reducing agent DTT, which does not cleave the peptide from the resin.⁴² I then treated resin with N-Chlorosuccinimide to oxidize the cysteine residues and then washed it out with DMF and DCM. The normal procedure was then followed to cleave the peptide from the resin. This procedure worked well and no contaminants showed up in the assays or on mass spectrometry. The only problem was that while oxidizing on resin worked well for peptide I, peptides II and III contain homocysteine residues, which is not available as an Fmoc amino acid with an S-Tmp protecting group. We decided to synthesize these peptides one final time and oxidize them as we did before with the Clear-Ox resin. As expected, there was some TNB contamination following the oxidation. To solve this problem we passed the peptide solution over a DEAE resin, an anion exchange resin that binds the negatively charged TNB contaminants and lets the positively charged peptides flow through. This was done multiple times until all of the peptide solution ran clear and no characteristic TNB/DTNB peak at 412 nm was visible on the spectrophotometer. The assays of peptides II and III were successful following this treatment.

Conclusion.

I have investigated the C-terminal redox center of PfTR through peptide substrate assays and semisynthesis. My results provide evidence that Cys⁵⁴⁰ is attacked by the N-terminal redox center to initiate intramolecular thiol-disulfide exchange. This reaction is made favorable by an electrophilic activation mechanism on the Cys⁵³⁵-Cys⁵⁴⁰ disulfide bond through hydrogen bonding with His⁵⁰⁹, resulting in N-terminal attack on Cys⁵⁴⁰. The substitution of Cys⁵³⁵ with Sec results in catalytic rate reduction, which I contend is evidence of a “seleno-effect” that inhibits His⁵⁰⁹ hydrogen bonding and inhibits electrophilic activation. My results also demonstrate the importance of the disulfide ring size on this catalytic mechanism as well as multiple methods for oxidation of peptide disulfide bonds that prevent oxidant contamination.

REFERENCES

- [1] Ingles-Prieto, A., Ibarra-Molero, B., Delgado-Delgado, A., Perez-Jimenez, R., Fernandez, J. M., Gaucher, E. A., Sanchez-Ruiz, J. M., and Gavira, J. A. (2013) Conservation of protein structure over four billion years, *Structure (London, England : 1993)* 21, 1690-1697.
- [2] Tonissen, K. F., and Wells, J. R. (1991) Isolation and characterization of human thioredoxin-encoding genes, *Gene* 102, 221-228.
- [3] Ago, T., and Sadoshima, J. (2006) Thioredoxin and Ventricular Remodeling, *Journal of molecular and cellular cardiology* 41, 762-773.
- [4] Holmgren, A. (1989) Thioredoxin and glutaredoxin systems, *The Journal of biological chemistry* 264, 13963-13966.
- [5] Hirota, K., Nishiyama, A., and Yodoi, J. (1999) [Reactive oxygen intermediates, thioredoxin, and Ref-1 as effector molecules in cellular signal transduction], *Tanpakushitsu kakusan koso. Protein, nucleic acid, enzyme* 44, 2414-2419.
- [6] Buchanan, B. B., Schurmann, P., and Jacquot, J. P. (1994) Thioredoxin and metabolic regulation, *Seminars in cell biology* 5, 285-293.
- [7] Holmgren, A. (1985) Thioredoxin, *Annual review of biochemistry* 54, 237-271.
- [8] Sevier, C. S., and Kaiser, C. A. (2002) Formation and transfer of disulphide bonds in living cells, *Nature reviews. Molecular cell biology* 3, 836-847.
- [9] Hagglund, P., Bunkenborg, J., Maeda, K., and Svensson, B. (2008) Identification of thioredoxin disulfide targets using a quantitative proteomics approach based on isotope-coded affinity tags, *Journal of proteome research* 7, 5270-5276.

- [10] Arner, E. S., and Holmgren, A. (2000) Physiological functions of thioredoxin and thioredoxin reductase, *European journal of biochemistry / FEBS* 267, 6102-6109.
- [11] Buchanan, B. B. (1991) Regulation of CO₂ assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. Perspective on its discovery, present status, and future development, *Archives of biochemistry and biophysics* 288, 1-9.
- [12] Hirota, K., Murata, M., Sachi, Y., Nakamura, H., Takeuchi, J., Mori, K., and Yodoi, J. (1999) Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB, *The Journal of biological chemistry* 274, 27891-27897.
- [13] Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1, *The EMBO journal* 17, 2596-2606.
- [14] Kang, S. W., Chae, H. Z., Seo, M. S., Kim, K., Baines, I. C., and Rhee, S. G. (1998) Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha, *The Journal of biological chemistry* 273, 6297-6302.
- [15] Laurent, T. C., Moore, E. C., and Reichard, P. (1964) ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEOTIDES. IV. ISOLATION AND CHARACTERIZATION OF THIOREDOXIN, THE HYDROGEN DONOR FROM ESCHERICHIA COLI B, *The Journal of biological chemistry* 239, 3436-3444.
- [16] Brot, N., and Weissbach, H. (1991) Biochemistry of methionine sulfoxide residues in proteins, *BioFactors* 3, 91-96.

- [17] Lehninger, A. L., Nelson, D. L., and Cox, M. M. (2013) *Lehninger principles of biochemistry*, 6th ed., W.H. Freeman, New York.
- [18] Gasdaska, P. Y., Gasdaska, J. R., Cochran, S., and Powis, G. (1995) Cloning and sequencing of a human thioredoxin reductase, *FEBS letters* 373, 5-9.
- [19] Fritz-Wolf, K., Urig, S., and Becker, K. (2007) The structure of human thioredoxin reductase 1 provides insights into C-terminal rearrangements during catalysis, *Journal of molecular biology* 370, 116-127.
- [20] Lothrop, A. P., Snider, G. W., Ruggles, E. L., and Hondal, R. J. (2014) Why is mammalian thioredoxin reductase 1 so dependent upon the use of selenium?, *Biochemistry* 53, 554-565.
- [21] Hondal, R. J., Marino, S. M., and Gladyshev, V. N. (2013) Selenocysteine in thiol/disulfide-like exchange reactions, *Antioxidants & redox signaling* 18, 1675-1689.
- [22] Finlayson, A. J., MacKenzie, S. L., and Finley, J. W. (1979) Reaction of alanine-3-sulfinic acid with 2-mercaptoethanol, *Canadian Journal of Chemistry* 57, 2073-2077.
- [23] Snider, G. W., Ruggles, E., Khan, N., and Hondal, R. J. (2013) Selenocysteine confers resistance to inactivation by oxidation in thioredoxin reductase: comparison of selenium and sulfur enzymes, *Biochemistry* 52, 5472-5481.
- [24] Eckenroth, B. E., Rould, M. A., Hondal, R. J., and Everse, S. J. (2007) Structural and biochemical studies reveal differences in the catalytic mechanisms of mammalian and *Drosophila melanogaster* thioredoxin reductases, *Biochemistry* 46, 4694-4705.
- [25] Snider, G. W., Dustin, C. M., Ruggles, E. L., and Hondal, R. J. (2014) A mechanistic investigation of the C-terminal redox motif of thioredoxin reductase from *Plasmodium falciparum*, *Biochemistry* 53, 601-609.

- [26] Kanzok, S. M., Fechner, A., Bauer, H., Ulschmid, J. K., Muller, H. M., Botella-Munoz, J., Schneuwly, S., Schirmer, R., and Becker, K. (2001) Substitution of the thioredoxin system for glutathione reductase in *Drosophila melanogaster*, *Science* 291, 643-646.
- [27] Arscott, L. D., Gromer, S., Schirmer, R. H., Becker, K., and Williams, C. H. (1997) The mechanism of thioredoxin reductase from human placenta is similar to the mechanisms of lipoamide dehydrogenase and glutathione reductase and is distinct from the mechanism of thioredoxin reductase from *Escherichia coli*, *Proceedings of the National Academy of Sciences of the United States of America* 94, 3621-3626.
- [28] Lothrop, A. P., Snider, G. W., Ruggles, E. L., Patel, A. S., Lees, W. J., and Hondal, R. J. (2014) Selenium as an electron acceptor during the catalytic mechanism of thioredoxin reductase, *Biochemistry* 53, 654-663.
- [29] Gromer, S., Johansson, L., Bauer, H., Arscott, L. D., Rauch, S., Ballou, D. P., Williams, C. H., Jr., Schirmer, R. H., and Arner, E. S. (2003) Active sites of thioredoxin reductases: why selenoproteins?, *Proceedings of the National Academy of Sciences of the United States of America* 100, 12618-12623.
- [30] Lothrop, A. P., Snider, G. W., Flemer, S., Jr., Ruggles, E. L., Davidson, R. S., Lamb, A. L., and Hondal, R. J. (2014) Compensating for the absence of selenocysteine in high-molecular weight thioredoxin reductases: the electrophilic activation hypothesis, *Biochemistry* 53, 664-674.
- [31] Krnajski, Z., Gilberger, T. W., Walter, R. D., and Muller, S. (2001) The malaria parasite *Plasmodium falciparum* possesses a functional thioredoxin system, *Molecular and biochemical parasitology* 112, 219-228.

- [32] Bauer, H., Massey, V., Arscott, L. D., Schirmer, R. H., Ballou, D. P., and Williams, C. H., Jr. (2003) The mechanism of high Mr thioredoxin reductase from *Drosophila melanogaster*, *The Journal of biological chemistry* 278, 33020-33028.
- [33] Breslow, R., and Chapman, W. H., Jr. (1996) On the mechanism of action of ribonuclease A: relevance of enzymatic studies with a p-nitrophenylphosphate ester and a thiophosphate ester, *Proceedings of the National Academy of Sciences of the United States of America* 93, 10018-10021.
- [34] Yoshinari, K., and Taira, K. (2000) A further investigation and reappraisal of the thio effect in the cleavage reaction catalyzed by a hammerhead ribozyme, *Nucleic Acids Research* 28, 1730-1742.
- [35] Ora, M., Lönnberg, T., and Lönnberg, H. (2012) Thio Effects as a Tool for Mechanistic Studies of the Cleavage of RNA Phosphodiester Bonds: The Chemical Basis, In *From Nucleic Acids Sequences to Molecular Medicine* (Erdmann, V. A., and Barciszewski, J., Eds.), pp 47-65, Springer Berlin Heidelberg.
- [36] Hondal, R. J., Bruzik, K. S., Zhao, Z., and Tsai, M.-D. (1997) Mechanism of Phosphatidylinositol-Phospholipase C. 2. Reversal of a Thio Effect by Site-Directed Mutagenesis¹, *Journal of the American Chemical Society* 119, 5477-5478.
- [37] Platts, J. A., Howard, S. T., and Bracke, B. R. F. (1996) Directionality of Hydrogen Bonds to Sulfur and Oxygen, *Journal of the American Chemical Society* 118, 2726-2733.
- [38] Flemer, S., Jr. (2015) Fmoc-Sec(Xan)-OH: synthesis and utility of Fmoc selenocysteine SPPS derivatives with acid-labile sidechain protection, *Journal of peptide science : an official publication of the European Peptide Society* 21, 53-59.

- [39] Evans, T. C., Jr., Benner, J., and Xu, M. Q. (1998) Semisynthesis of cytotoxic proteins using a modified protein splicing element, *Protein science : a publication of the Protein Society* 7, 2256-2264.
- [40] Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides, *Analytical biochemistry* 34, 595-598.
- [41] Han, Y., Albericio, F., and Barany, G. (1997) Occurrence and Minimization of Cysteine Racemization during Stepwise Solid-Phase Peptide Synthesis(1),(2), *J Org Chem* 62, 4307-4312.
- [42] Postma, T. M., Giraud, M., and Albericio, F. (2012) Trimethoxyphenylthio as a highly labile replacement for tert-butylthio cysteine protection in Fmoc solid phase synthesis, *Organic letters* 14, 5468-5471.
- [43] Steinmann, D., Nauser, T., and Koppenol, W. H. (2010) Selenium and sulfur in exchange reactions: a comparative study, *J Org Chem* 75, 6696-6699.
- [44] Eckenroth, B. E., Lacey, B. M., Lothrop, A. P., Harris, K. M., and Hondal, R. J. (2007) Investigation of the C-terminal redox center of high-Mr thioredoxin reductase by protein engineering and semisynthesis, *Biochemistry* 46, 9472-9483.
- [45] Lacey, B. M., Eckenroth, B. E., Flemer, S., and Hondal, R. J. (2008) Selenium in thioredoxin reductase: a mechanistic perspective, *Biochemistry* 47, 12810-12821.
- [46] Lobanov, A. V., Delgado, C., Rahlfs, S., Novoselov, S. V., Kryukov, G. V., Gromer, S., Hatfield, D. L., Becker, K., and Gladyshev, V. N. (2006) The Plasmodium selenoproteome, *Nucleic Acids Res* 34, 496-505.

- [47] Nauser, T., Steinmann, D., Grassi, G., and Koppenol, W. H. (2014) Why selenocysteine replaces cysteine in thioredoxin reductase: a radical hypothesis, *Biochemistry* 53, 5017-5022.
- [48] Kanzok, S. M., Schirmer, R. H., Turbachova, I., Iozef, R., and Becker, K. (2000) The thioredoxin system of the malaria parasite *Plasmodium falciparum*. Glutathione reduction revisited, *The Journal of biological chemistry* 275, 40180-40186.

TABLES

Table 1: Activities of synthesized peptides assayed with PfTR Δ 7

Peptide #	Peptide Sequence	Ring size	K_{cat} (min^{-1})	K_m (mM)	Specific activity at 5 mM peptide (mol NADPH/min/mol TR)
I	GC ⁵³⁵ GGGKC ⁵⁴⁰ G	20	320 \pm 30	4.8 \pm 0.9	167 \pm 4
II	GCGGGK h CG	21	^a	^a	31 \pm 3
III	G h CGGGK C CG	21	155 \pm 12	12 \pm 1	47 \pm 11
IV	GCGGGK U G	20	^a	^a	289 \pm 36
V	G U GGGK C CG	20	114 \pm 32	12 \pm 4.6	36 \pm 18

^aThese assays resulted in linear trends not suitable to plot with Michaelis-Menten

Table 2: Kinetics of semisynthetic enzymes with Trx and selenocystine

Ligated Enzyme	Sequence	Trx K_{cat} (min^{-1})	Trx K_M (μM)	Sec ² K_{cat} (min^{-1})	Sec ² K_M (μM)
A	PfTR-CGGGK U G	561 \pm 39	18 \pm 5	672 \pm 73	84 \pm 25
B	PfTR- U GGGK C CG	7.3 \pm 0.5	9.3 \pm 3	24 \pm 1	68 \pm 13

FIGURES

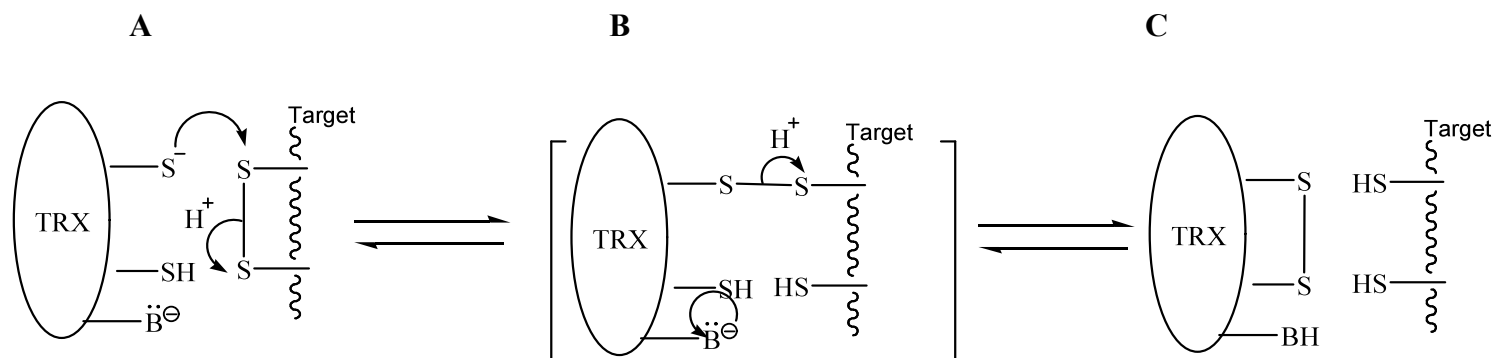


Figure 1: Thiol-disulfide exchange between Trx and a target protein. A) A reduced Trx cysteine residue in the active site attacks the oxidized disulfide of a target protein. B) Formation of a mixed disulfide transition state between Trx and its target. C) Exchange is complete; the target is left reduced while Trx has formed an oxidized disulfide between its active site cysteine residues.

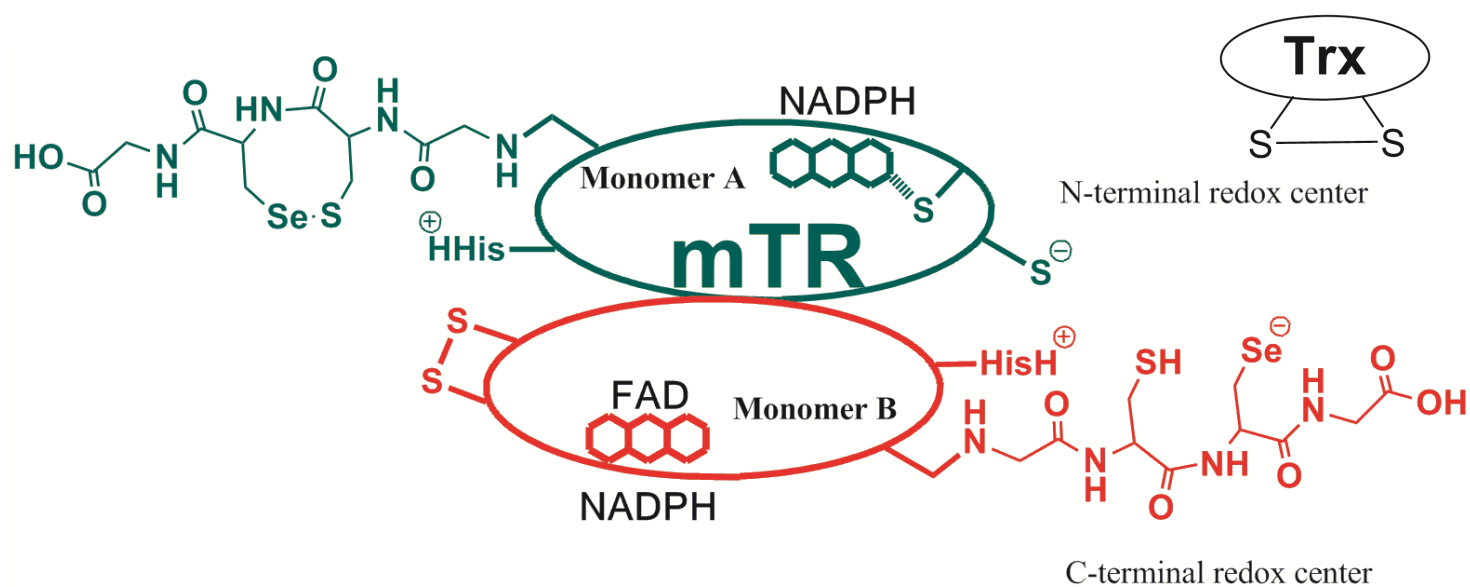


Figure 2: Mammalian TR homodimer with redox centers shown. High MW TR enzymes contain three redox centers in each monomer; a bound flavin domain, an N-terminal disulfide redox center and a C-terminal disulfide redox center, as well as an NADPH binding domain. Electrons flow through these redox centers to ultimately allow the C-terminal redox center to reduce the substrate Trx. First, NADPH bind to its binding domain and reduces the flavin domain. This flavin then reduces the disulfide of the N-terminal redox center. This redox center then reduces the C-terminal redox center of the opposite monomer through thiol disulfide exchange. In this depiction the right side of the enzyme is shown in the reduced configuration while the left side is shown oxidized.

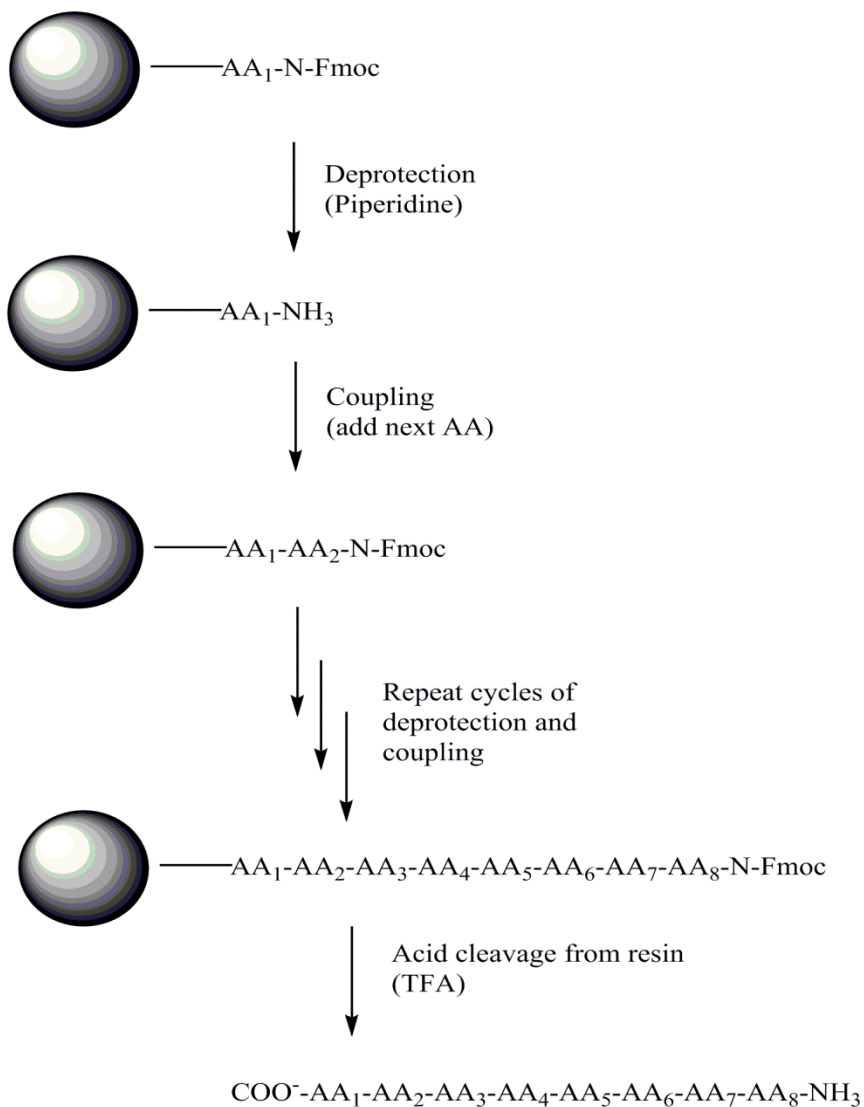


Figure 3: General solid phase Fmoc peptide synthesis procedure. Solid phase peptide synthesis is done on a resin that binds to the most C-terminal amino acid of the peptide. Each amino acid is protected by an Fmoc group on its amino end. After the first amino acid is coupled to the resin, this Fmoc group is deprotected using piperidine. The next amino acid in the sequence (C->N) is then added to the resin with a coupling reagent and a peptide bond is formed. This cycle of deprotection and coupling is repeated until the peptide sequence is complete. The peptide is then cleaved from the resin using strong acid and precipitated.

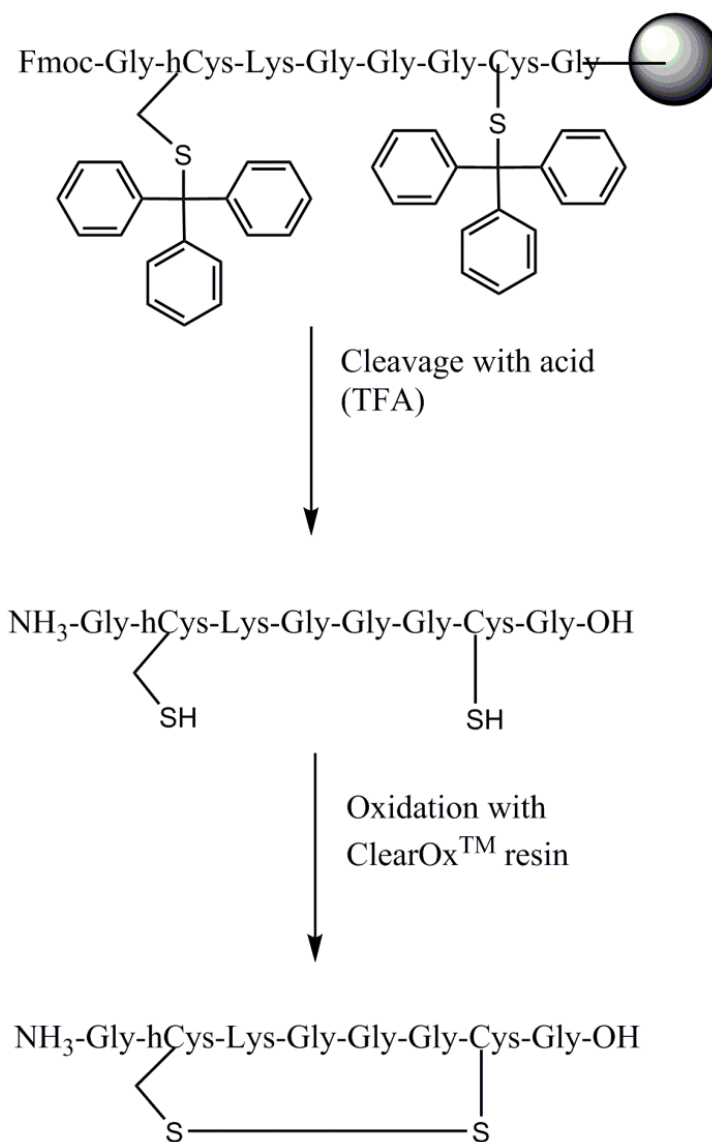


Figure 5: Off resin oxidation of homocysteine peptides (II and III). Cysteine and homocysteine are both protected by an acid labile Trt group. Upon cleavage of the peptide from the resin these groups were also removed. The peptides were then oxidized by passing them over a ClearOxTM resin with bound DTNB oxidizing agent. This formed a disulfide within the peptide, oxidizing it. Some TNB anions leaked off of the resin and contaminated the sample, so the sample was then run through an anion exchange resin that removed the contaminating anions.

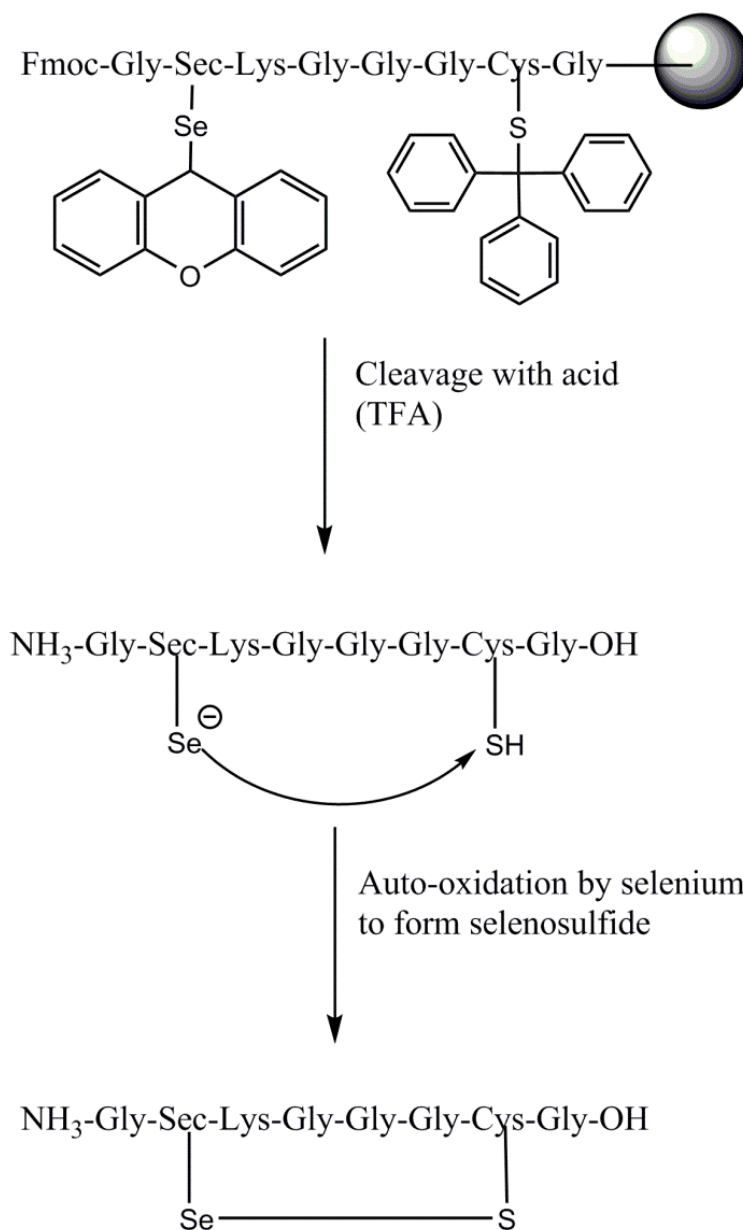


Figure 6: Off resin oxidation of selenocysteine peptides (IV and V). The cysteine residues were protected by an acid labile Trt group, while the selenocysteine residue was protected by an acid labile xanthene group. Upon cleavage of the peptide from the resin these groups were also removed. Selenium then reacted with free oxygen in the solution to auto-oxidize the peptide, forming a seleno-sulfide bond. Selenocysteine containing peptides were the easiest to oxidize because they do not need any oxidizing reagent other than free oxygen in solution.

PfTR-GC₁GGGKC₂G (WT)

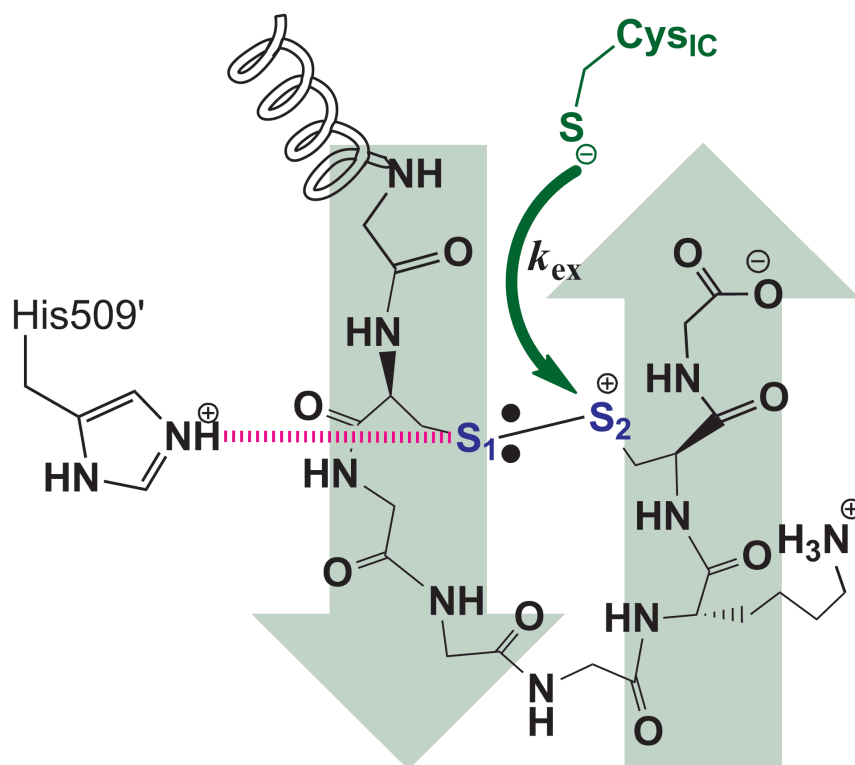


Figure 7: Proposed PfTR C-terminal redox center electrophilic activation mechanism. Hydrogen bonding between the positively charged His⁵⁰⁹ and the sulfur (S₁) of Cys⁵³⁵ (C₁) draws the electrons of the disulfide bond toward S₁, which then consequently induces a partial positive charge in the sulfur (S₂) of Cys⁵⁴⁰ (C₂). This makes S₂ more electrophilic and prone to attack by the negatively charged attacking cysteine residue of the N-terminal redox center (Cys_{1C}).

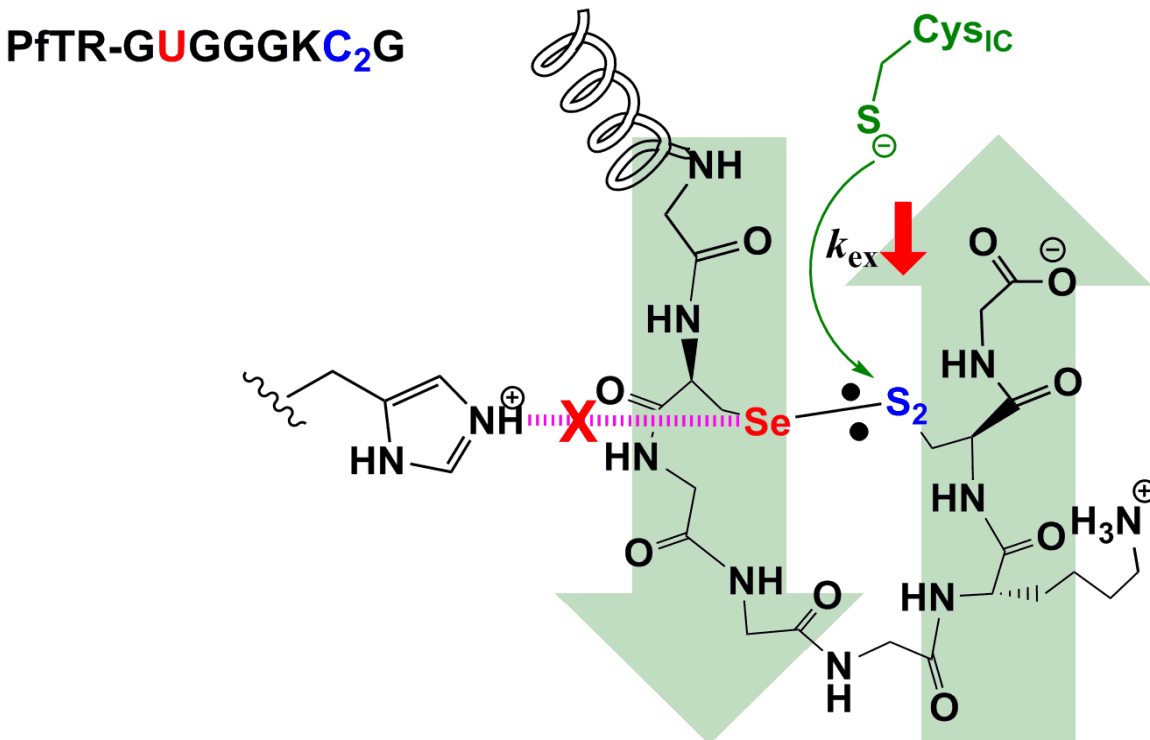


Figure 8: Mechanism of seleno effect rate reduction when Cys⁵³⁵ is substituted with Sec in C-terminal redox center of PfTR. When Cys⁵³⁵ is replaced with Sec the positive charge of His⁵⁰⁹ cannot induced a dipole within the selenosulfide bond. This is because selenium has a lower pKa than sulfur and will not hydrogen bond as readily to the histidine residue. The sulfur (S₂) of Cys⁵⁴⁰ (C₂) therefore no longer has an induced partial positive charge and is not a good electrophile for the attacking N-terminal cysteine residue (Cys_{1C}). This reduces the rate of thiol-disulfide exchange (k_{ex}) and the overall catalytic rate of PfTR.

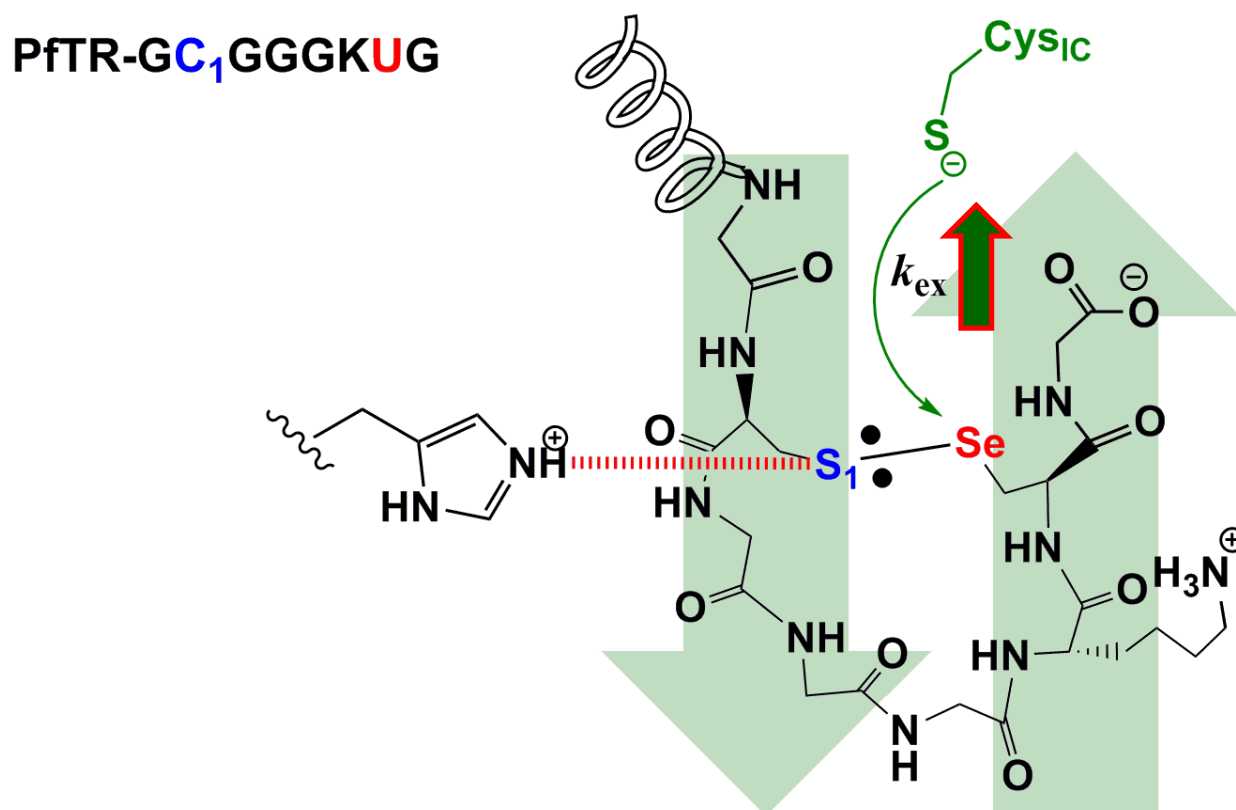


Figure 9: PfTR rate increase as a result of C-terminal redox center Cys⁵⁴⁰ to Sec substitution. Cys⁵⁴⁰ is the proposed residue that undergoes attack to initiate thiol disulfide exchange between the N-terminal and C-terminal redox centers. In this model Cys⁵⁴⁰ has been replaced by Sec. Selenium is a much better nucleophile than sulfur, making it a better target of attack for the N-terminal cysteine (Cys_{1C}). This substitution consequently results in an increase in rate for PfTR.

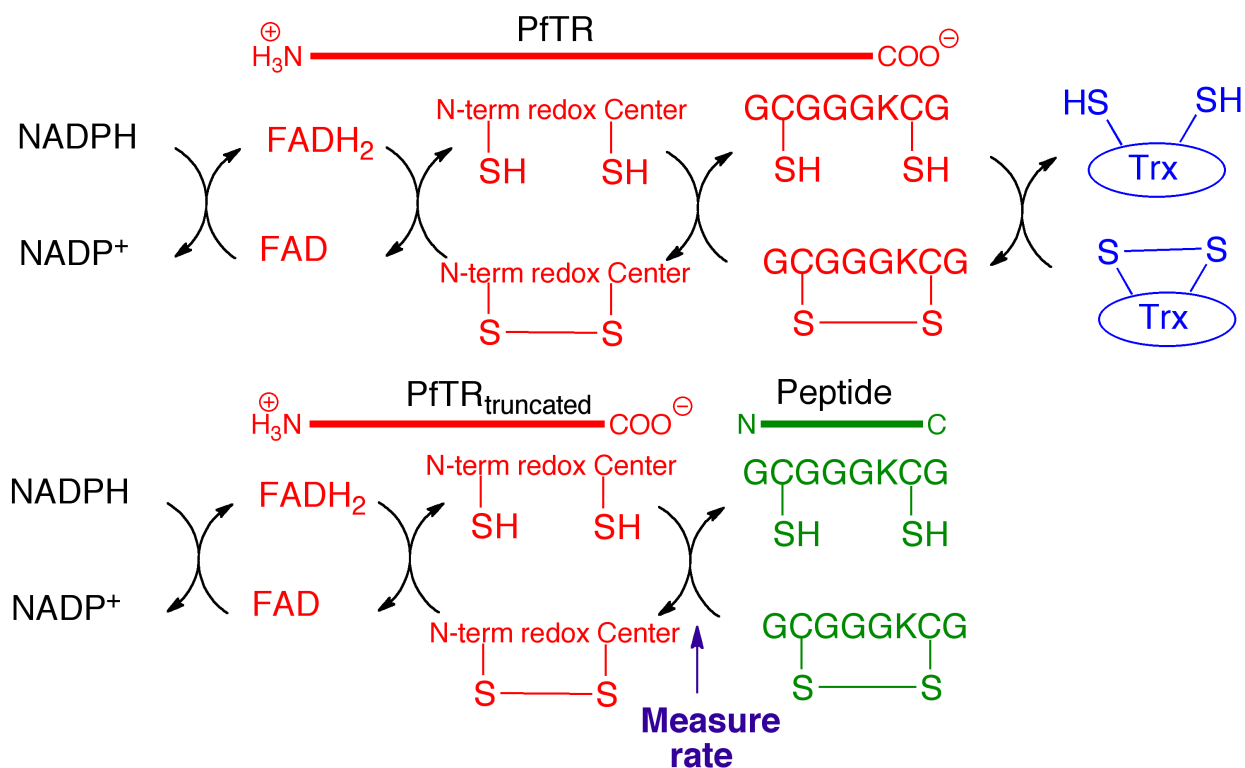


Figure 10: Redox cycle of full length PfTR with Trx as a substrate and PfTR Δ 7 with a peptide substrate. In order to isolate the exchange between the N-terminal and C-terminal redox centers PfTR Δ 7 was assayed with a peptide substrate with the structure of the C-terminal redox center. This allowed the consumption of NADPH to directly reflect this redox exchange. In the full length enzyme this exchange is intramolecular and Trx is the substrate being reduced.

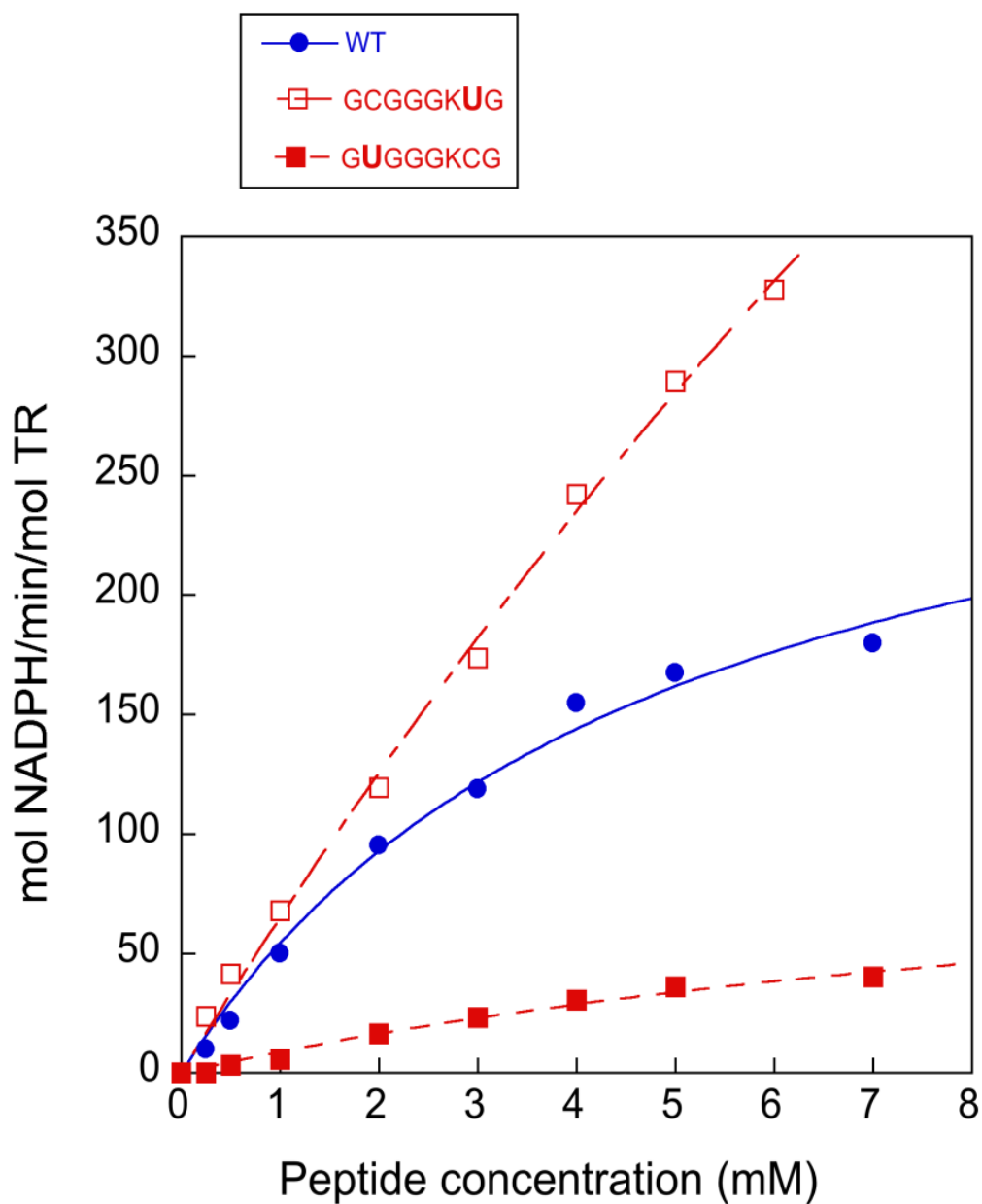


Figure 11: Enzyme assays of peptides 1, 4, and 5 with PfTRΔ7. This Michaelis-Menten plot shows the K_{cat} of PfTRΔ7 with peptides 1, 4, and 5 as substrates plotted against peptide concentration. These illustrate the difference in Sec substitution at the attacked Cys⁵⁴⁰, resulting in an increase in activity, versus a Sec substitution at the activating Cys⁵³⁵, resulting in a decrease due to the disturbance of electrophilic activation by the seleno-effect. These are plotted with the wild type peptide to show relative activity.

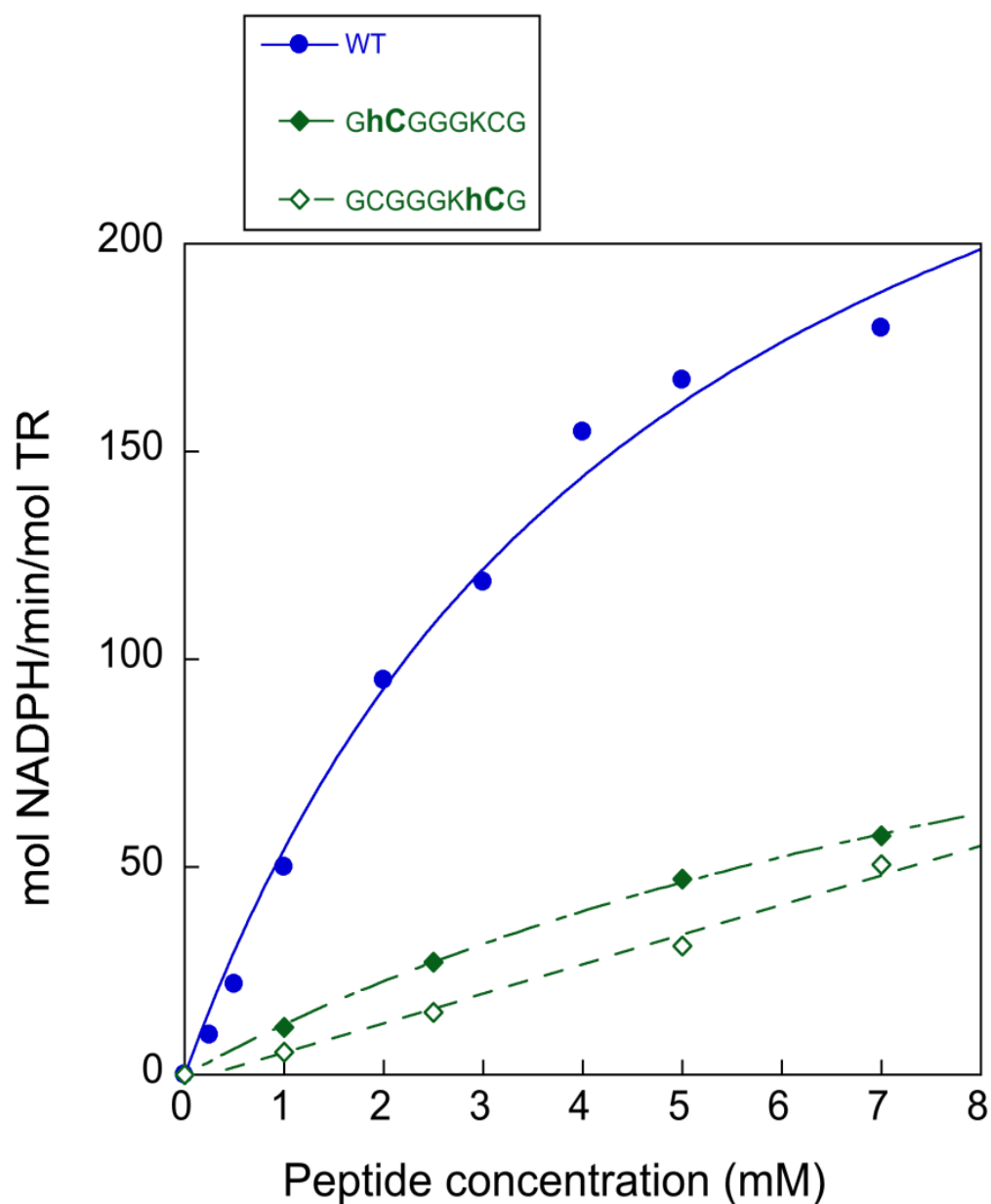


Figure 12: Enzyme assays of peptides 1, 2, and 3 with PfTRΔ7. The Michaelis-Menten plot shows the K_{cat} of PfTRΔ7 with peptides 1, 2, and 3 as substrates plotted against peptide concentration. This shows the difference in activity between the wild type peptide and the two homocysteine mutant peptides. Homocysteine substitution at either location results in a similar reduction in PfTRΔ7 activity.

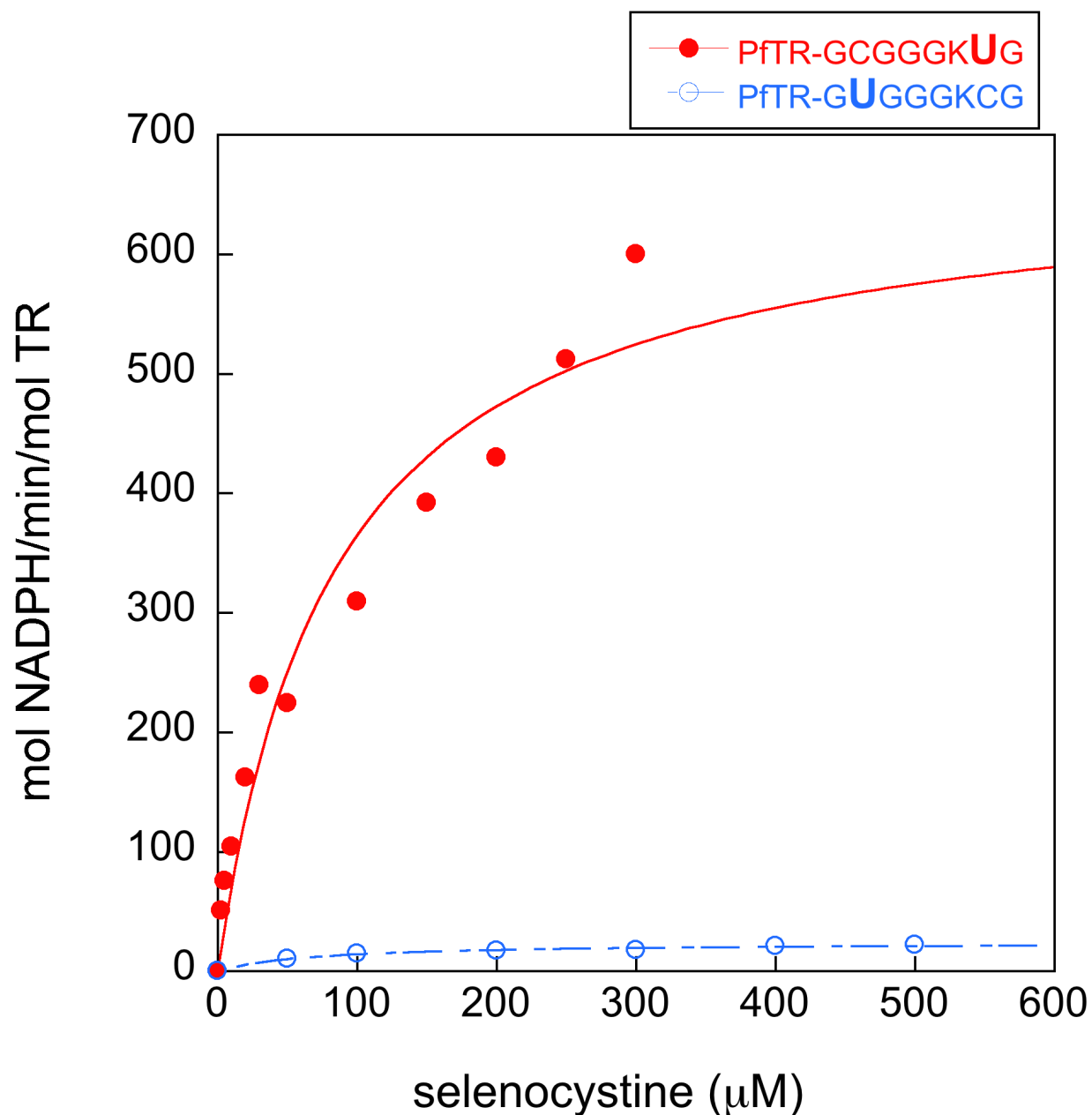


Figure 13: Enzyme assay plot of ligated enzymes A and B with selenocystine as a substrate. These Michealis-Menten curves show the how Sec for Cys substitution increases enzyme activity towards selenocystine at Cys⁵⁴⁰ and decreases it at Cys⁵³⁵. This is due to the increased electrophilicity of selenium over sulfur when acting as an electrophile at Cys⁵⁴⁰ and the inhibition of PfTR's electrophilic activation mechanism through the seleno effect at Cys⁵³⁵. PfTR-GCGGGKUG has approximately 30 fold greater activity than PFTR-GUGGGKCG.

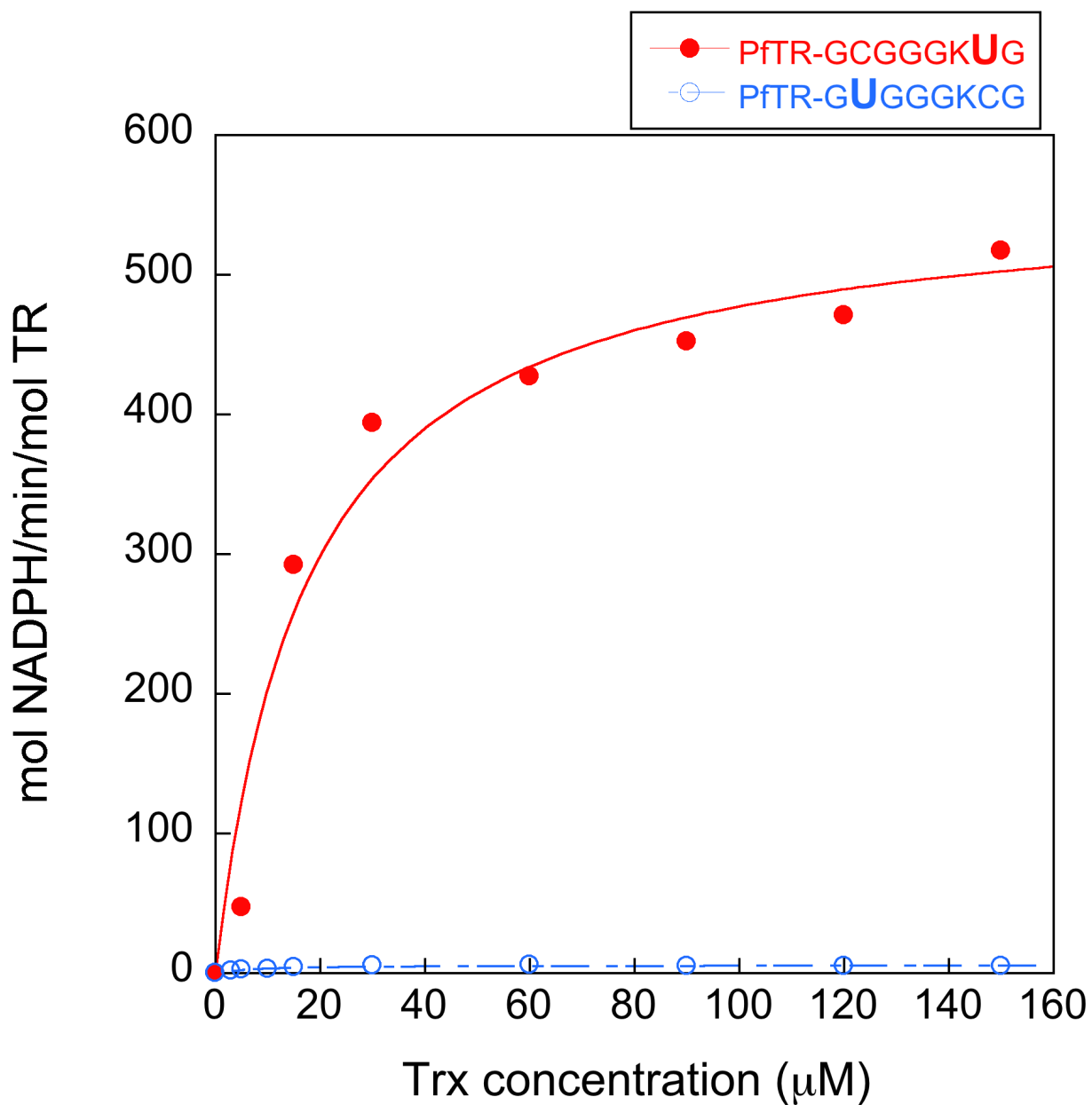


Figure 14: Enzyme assay plot of ligated enzymes A and B with thioredoxin as a substrate. The Michaelis-Menten curves show how enzyme activity following Cys⁵⁴⁰→Sec substitution is much greater than the activity following Cys⁵³⁵→Sec substitution with Trx as a substrate. PfTR-GCGGGKUG has approximately 75 fold greater activity than PfTR-GUGGGKCG.

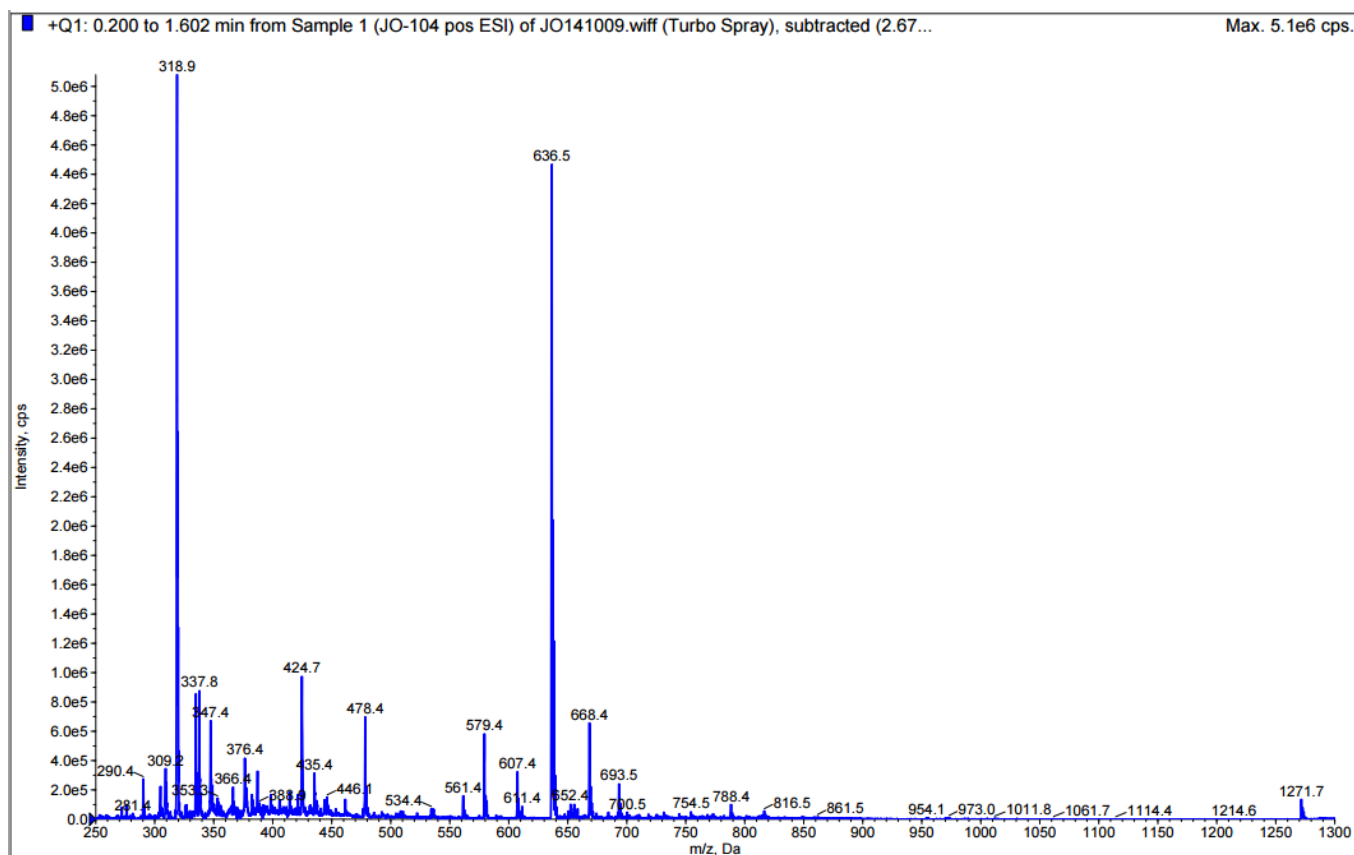


Figure 15: Mass spectral analysis of Peptide I. The molecular weight of Peptide I when oxidized is 636 (MW = 638 when reduced). The sample was analyzed through mass spectrometry using direct infusion in positive ESI. The plot shows peaks at both the MW and one half of the MW (318) for the doubly charged peptide.

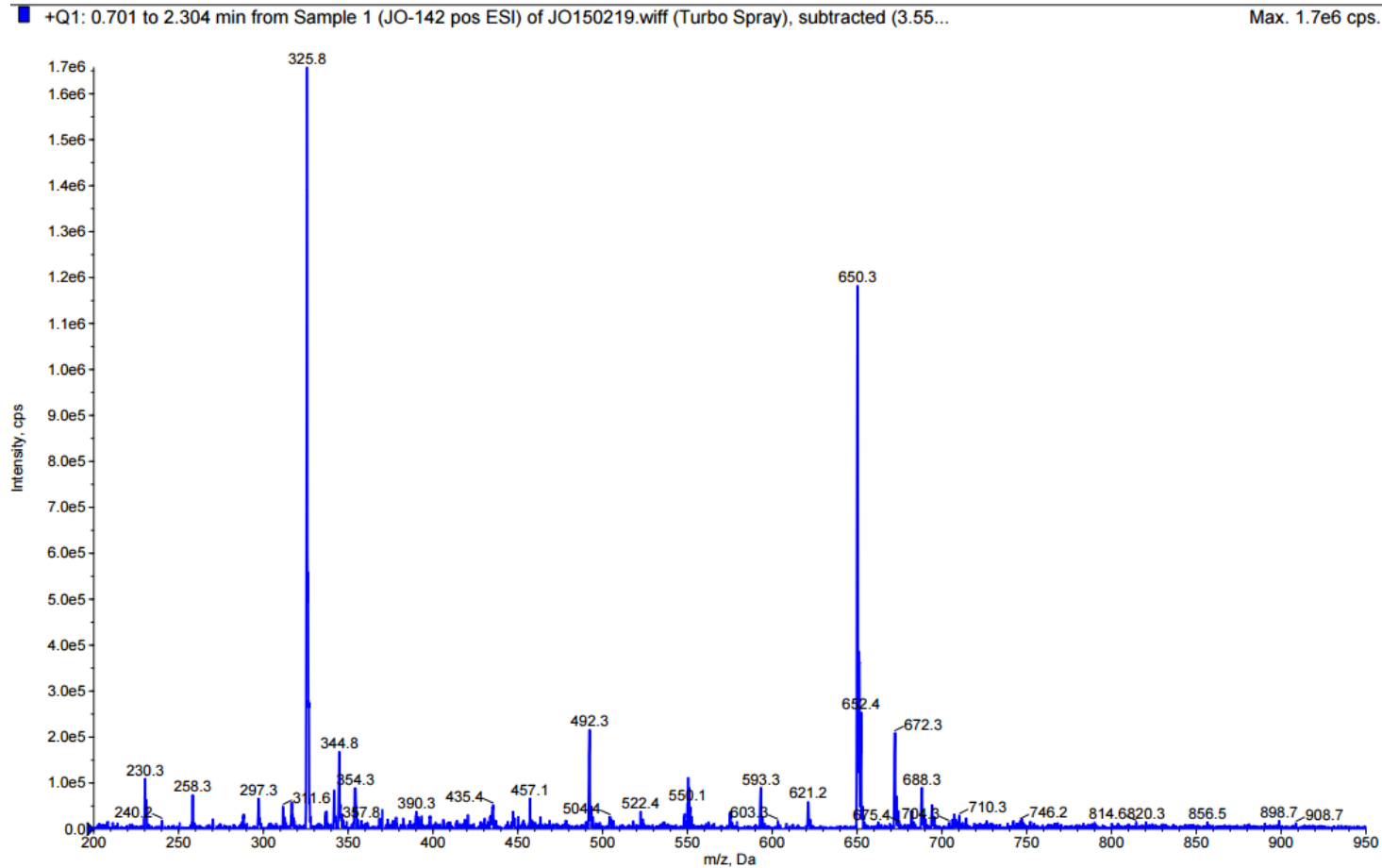


Figure 16: Mass spectral analysis of Peptide II. The molecular weight of Peptide II when oxidized is 650 (MW = 652 when reduced). The sample was analyzed through mass spectrometry using direct infusion in positive ESI. The plot shows peaks at both the MW and one half of the MW (325) for the doubly charged peptide.

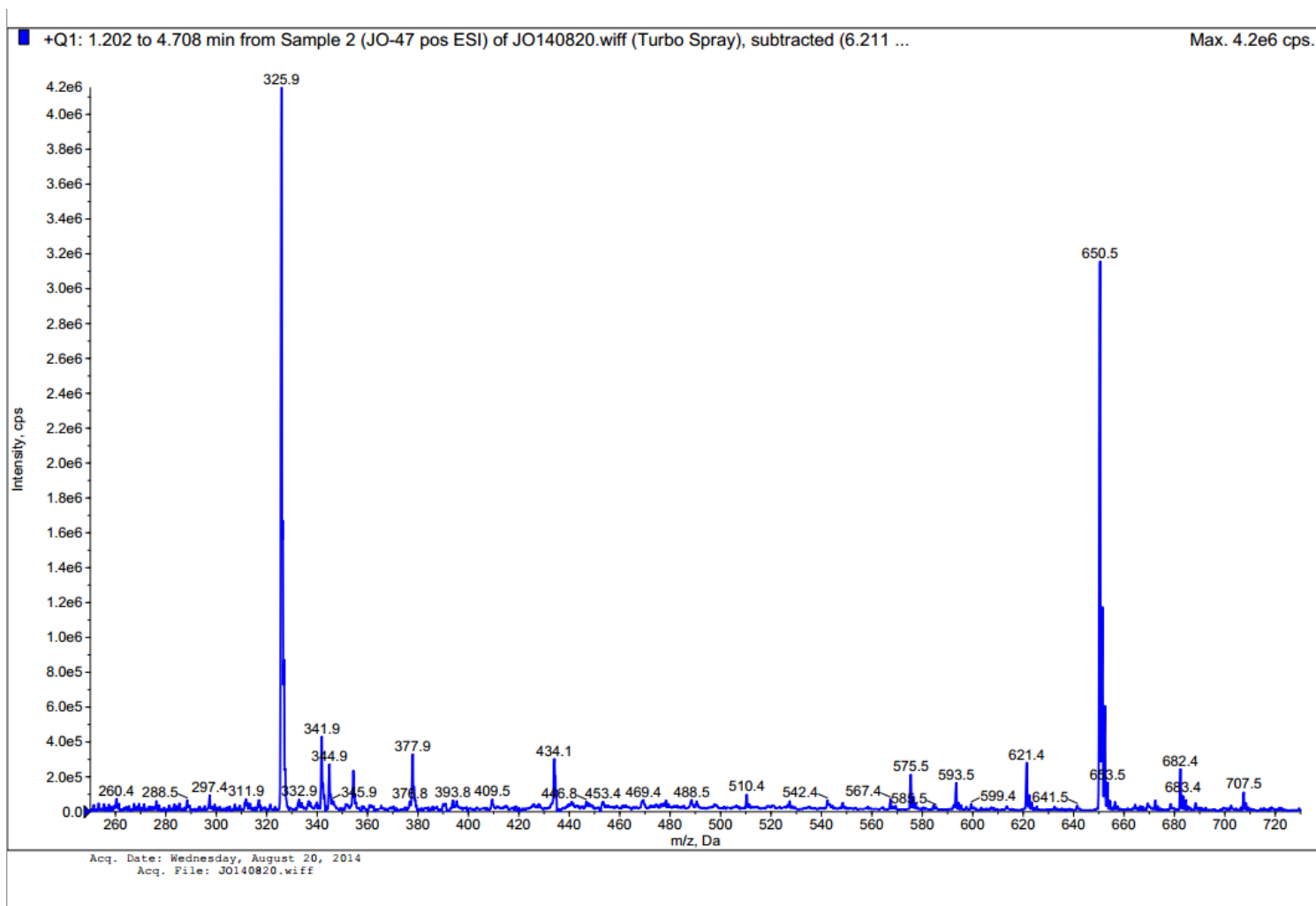


Figure 17: Mass spectral analysis of Peptide III. The molecular weight of Peptide III when oxidized is 650 (MW = 652 when reduced). The sample was analyzed through mass spectrometry using direct infusion in positive ESI. The plot shows peaks at both the MW and one half of the MW (325) for the doubly charged peptide.

+Q1: 0.125 to 0.960 min from Sample 12 (CMD 6-73 peak 3 pos ESI) of CD140701.wiff (Turbo Spray), subtr...

Max. 4.4e6 cps.

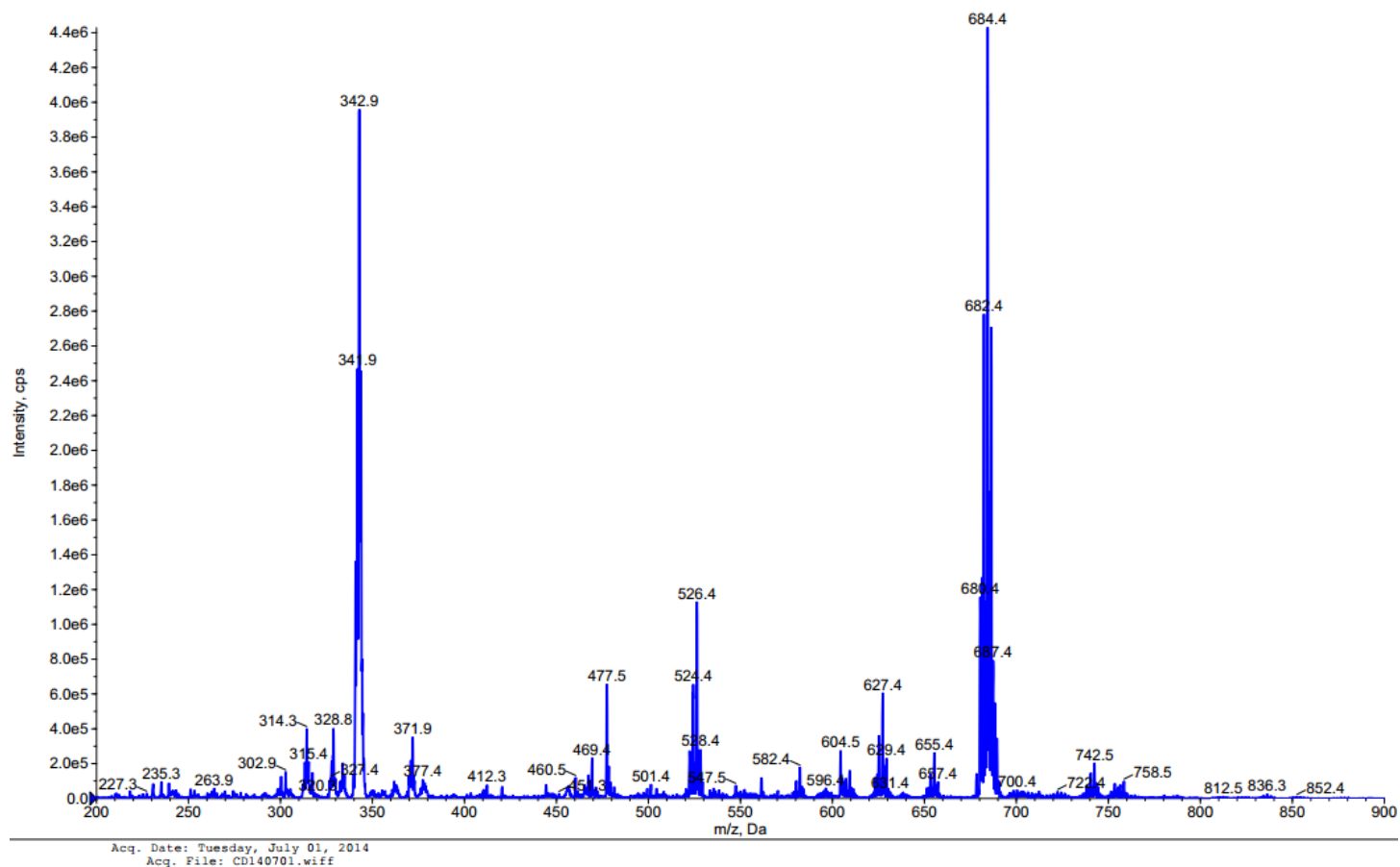


Figure 18: Mass spectral analysis of Peptide IV. The molecular weight of Peptide IV when oxidized is 684 (MW = 686 when reduced). The sample was analyzed through mass spectrometry using direct infusion in positive ESI. The plot shows peaks at both the MW and one half of the MW (342) for the doubly charged peptide.

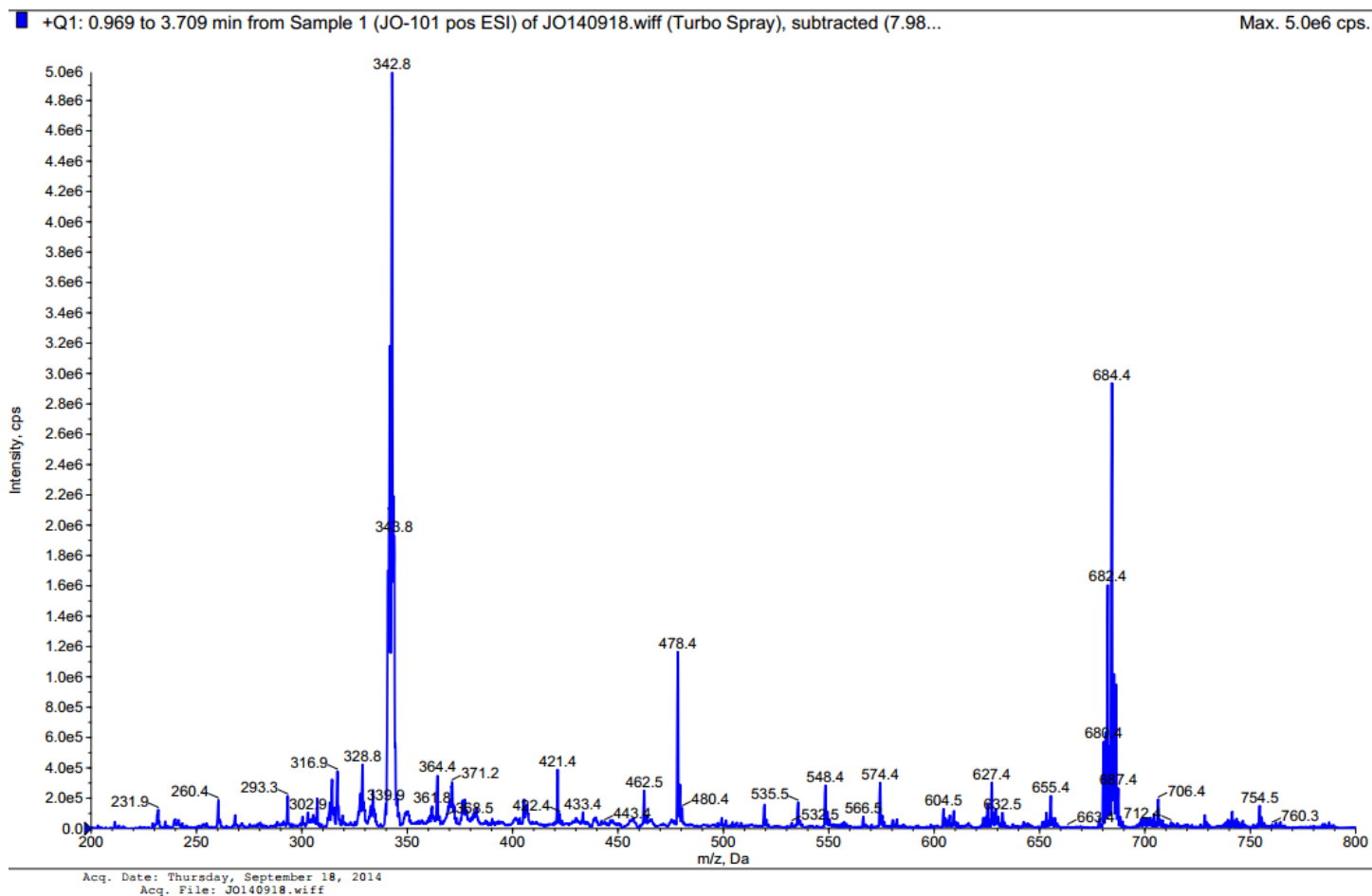


Figure 19: Mass spectral analysis of Peptide V. The molecular weight of Peptide V when oxidized is 684 (MW = 686 when reduced). The sample was analyzed through mass spectrometry using direct infusion in positive ESI. The plot shows peaks at both the MW and one half of the MW (342) for the doubly charged peptide.