2023

Ergothioneine in an Enzyme: Using Protein Engineering to Create Unique Antioxidant Enzymes Containing 2-thiohistidine

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Ergothioneine in an Enzyme: Using Protein Engineering to Create Unique Antioxidant Enzymes Containing 2-thiohistidine

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Abstract:

Ergothioneine is a sulfur containing amino acid found in foods such as mushrooms. It is a unique antioxidant and is a potential vitamin. However, ergothioneine is unable to be incorporated into a peptide or protein because its nitrogen is trimethylated. The amino acid 2-thiohistidine is an analogue of ergothioneine with similar antioxidant properties but can be inserted into a peptide/protein because the nitrogen is bonded to three hydrogens instead of three carbons. The goal of this project is to use protein engineering to replace a catalytic cysteine residue of the C-terminus of *Plasmodium falciparum* thioredoxin reductase (PfTrxR) with 2-thiohistidine in three variants: PfTrxR–CGGGK2THG, PfTrxR– CG2THKPG2THK, and PfTrxR– CUGGK2THG, where "PfTrxR" represents the body of the enzyme and the letters after the dash are the amino acids of the C-terminal redox center. PfTrxR–CGGGK2THG was hypothesized to gain the ability to reduce free radicals; PfTrxR– CG2THKPG2THK to metabolize singlet oxygen, and the 2TH in PfTrxR–CUGGK2THG to protect the active selenocysteine residue. The catalytic cysteine of PfTrxR is also a sulfur-containing amino acid, but it reduces radicals very poorly. Our data supports the protective effects of 2-thiohistidine on the redox-active residue selenocysteine. It was also observed that the mutant PfTrxR–CUGGK2THG may have gained the ability to metabolize singlet oxygen, instead of PfTrxR–CG2THKPG2THK. This work is an example of protein engineering using a non-standard amino acid in which the mutant gained a new function that was not possible using only the twenty standard amino acids.

Introduction: Ergothioneine (EGT) is a sulfur containing amino acid derivative of histidine. It is synthesized naturally in fungi and bacteria from the amino acids cysteine and histidine (**Figure 1**) but must be taken in through food in vertebrates. EGT is hydrophilic and therefore does not easily cross cell membranes and requires the EGT transporter (ETT). ETT is highly expressed in certain tissues in humans, including erythrocyte progenitor cells in the bone marrow, the small intestine, kidney, trachea, monocytes, and the cerebellum[1]. EGT displays antioxidant activity in the body through two mechanisms: (i) quenching free radicals, which results in oxidation of the sulfur group present on EGT to produce a disulfide and, (ii) reduction of singlet oxygen ($^1O_2$), an excited form of oxygen that forms in the presence of light and a photosensitizer such as a pigment[2] and other naturally occurring processes (**Figure 1**). Ergothioneine has been shown to

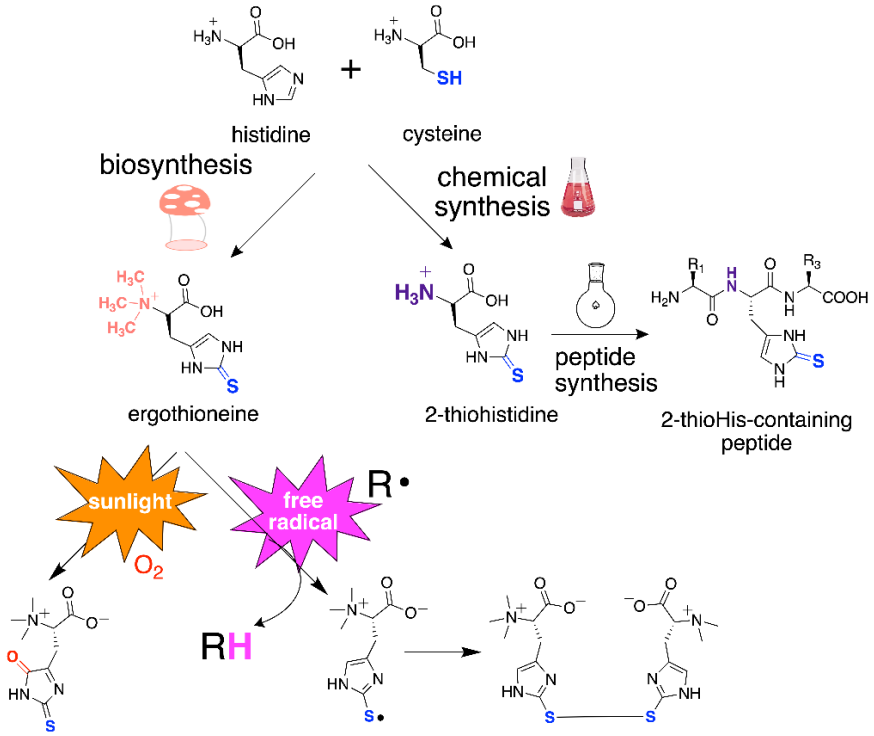

Figure 1: **Sources of ergothioneine (EGT) and 2-thiohistidine.** EGT is *biosynthesized* by fungi and bacteria from the amino acids histidine and cysteine. We *chemically synthesize* 2-thioHis, an EGT analogue, from the same amino acids. 2-thioHis has the same chemical properties as EGT but can be inserted into a peptide/protein due to differences in bonding at N. EGT and 2-thiohistidine act as antioxidants by either quenching free radicals (R•) or singlet oxygen. When EGT reacts with a radical, an EGT radical forms, which reacts with a second EGT radical to form the disulfide as shown here. Singlet oxygen is an excited form of oxygen that forms when sunlight reacts with oxygen in the presence of a pigment that acts as a photosensitizer.

be a scavenger of many free radicals and reactive oxygen species found in the body including the hydroxyl radical ($^{\cdot}OH$)[3], hypochlorous acid (HOCl)[2], and peroxynitrite[4].

Singlet oxygen is a reactive oxygen species that reacts with a variety of molecules in the body, including DNA, RNA, lipids, proteins, and sterols. Its reaction with unsaturated fatty acids in the body produces lipid peroxides, and oxidation of sterols such as cholesterol results in the production of mixtures of hydroperoxides. Additionally, as is pertinent to this project, $^1O_2$ oxidizes compounds that contain heteroatoms such as sulfur and nitrogen, forming sulfoxides and *N*-oxides[5]. EGT contains the heteroatom sulfur, and therefore is a target of $^1O_2$. This, among the formation of other oxidized species in the body results in oxidative stress, a large component of multifactorial diseases related to ageing such as cardiovascular disease, chronic kidney disease, and neurodegenerative diseases, among others[6]. One important example of singlet oxygen reactivity is its reaction with the nucleobase guanine[7]. The oxidation of guanine by singlet oxygen results in the formation of various reaction products, one of the most commonly studied being 8-oxo-guanine. The oxidation of guanine by $^1O_2$ results in mutagenesis of DNA; most commonly G to T transversion mutations[8]. $^1O_2$ is generated in the body through many routes, but the main sources include reactions of light activating photosensitizers in the skin, reactions of hydrogen peroxide, and reactions of endoperoxides[5]. Overall singlet oxygen is a very important reactive oxygen species in the body.

EGT's reactivity with $^1O_2$ has been seen as a special reactivity of the molecule. EGT has been shown to quench $^1O_2$ at a greater rate than other sulfur containing antioxidants such as glutathione and ascorbate[9]. EGT can be regenerated non-enzymatically after reaction with singlet oxygen (**Figure 2**), and in a mechanistic study it was confirmed that the sulfur-containing ring provided the most activity with $^1O_2$, which supports previous data[10]. This study also found that

EGT and glutathione acted synergistically, with glutathione acting to regenerate EGT (**Figure 2**), and therefore may be viewed as a coupled system[10]. In a zebrafish model, EGT was shown to be protective against the oxidation of the nucleobase guanine to 8-oxoguanine by $^1O_2$. The zebrafish model was a knockout of the EGT transport protein, which resulted in a complete deficiency of EGT in the tissues of the fish. The investigators found a significant increase in the levels of 8-oxoguanine in the skin tissue of unstressed EGT transporter knockout fish, which is a result of greater oxidation of guanine by $^1O_2$[11]. EGT's unique reactivity with $^1O_2$ has been attributed to the five-membered imidazole-derivative ring present in EGT which is unique to its structure and gives EGT its niche function[11]. For these reasons, we predicted that the insertion of EGT into the C-terminal active site of the redox active enzyme thioredoxin reductase would confer new, unique activity.

Thioredoxin reductase (TrxR) enzymes, the protein thioredoxin (Trx), and NADPH make up the thioredoxin system[1]. TrxR is a flavoprotein which has a conserved active site at the C-terminus containing two cysteine residues which form a disulfide and act to reduce the protein disulfide of oxidized Trx, therefore acting as an antioxidant system. This is called a protein disulfide reductase, where NADPH donates electrons to TrxR, which then are used in the reduction of Trx back to its active state[1]. The thioredoxin system is widely conserved throughout nature, from eukaryotic to prokaryotic organisms. Each species has varying amino acid sequences at their C-terminal active site, which is where we are using semisynthesis in this project. For example, the mammalian TrxR contains a selenocysteine (Sec) residue in the C-terminus of the wild type enzyme with sequence Gly-Cys-Sec-Gly[1]. In contrast, the C-terminus of *Drosophila melanogaster* TrxR contains only a redox active disulfide with sequence Gly-Cys-**Cys**-Gly[12]. Finally, *Plasmodium falciparum* TrxR contains a seven amino acid long C-terminal active site which also

utilizes a disulfide formed between Cys residues (Cys-Gly-Gly-Gly-Lys-Cys-Gly)[13]. These differences in amino acid sequence, orientation, and length in the C-terminal active sites may be indicative of TrxR's niche of activity in the organism in which it is expressed.

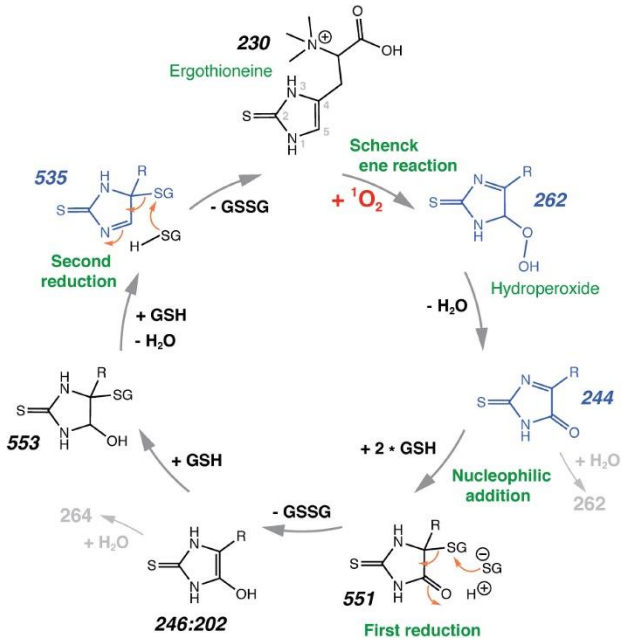

Figure 2: Proposed mechanism of EGT regeneration. Simplified mechanism of EGT regeneration as determined through LC/MS by Oumari et. al. Black species found in LC/MS, blue species are proposed intermediates, and grey species are possible side reactions[10]. Taken from Oumari et al.

Trx reduces various substrates in the cell[1], and the thioredoxin system is involved with many cellular processes. One important process is DNA replication and repair, where Trx reduces ribonucleotide reductase[1, 14]. This enzyme catalyzes deoxyribonucleotide synthesis. This is just one example of the effects of the thioredoxin system in a cell among many more such as in protein repair, cell cycle arrest, redox signaling, and apoptosis, among others[1, 15].

Recently, the Hondal group showed that thioredoxin reductase enzymes reduce the oxidized forms of EGT, such as the disulfide, returning EGT to its active, reduced state[16]. Wild-type TrxR enzymes cannot quench free radicals or $^1O_2$ directly. Free radicals are quenched by Trx which is then regenerated by TrxR. This inspired us to create a mutant TrxR that incorporated EGT into the amino acid sequence of the enzyme to give the enzyme new activity for quenching free radicals as illustrated in **Figure 3**. However, the problem with this idea is that EGT cannot be inserted into a peptide or protein due to its nitrogen being trimethylated (**Figure**

1), so its unique chemistry cannot be utilized directly in a protein. Instead, 2-thiohistidine (2-TH), an analogue of EGT, can be used because it has the same antioxidant properties of EGT, but is able to be inserted into a peptide due to its nitrogen not being trimethylated. 2-thiohistidine is not synthesized by living organisms and is therefore chemically synthesized in the lab from the amino acids cysteine and histidine.

The mutant enzymes were created through protein semisynthesis (protein engineering), which is used to join a small synthetic peptide with a larger polypeptide segment produced in bacteria using recombinant DNA technology. This allows for non-natural amino acids such as 2-thiohistidine to be put into the desired protein. This allows us to create a novel mutant enzyme that gains the unique antioxidant function of EGT (bottom panel of **Figure 3** and described in more detail in **Figure 4**).

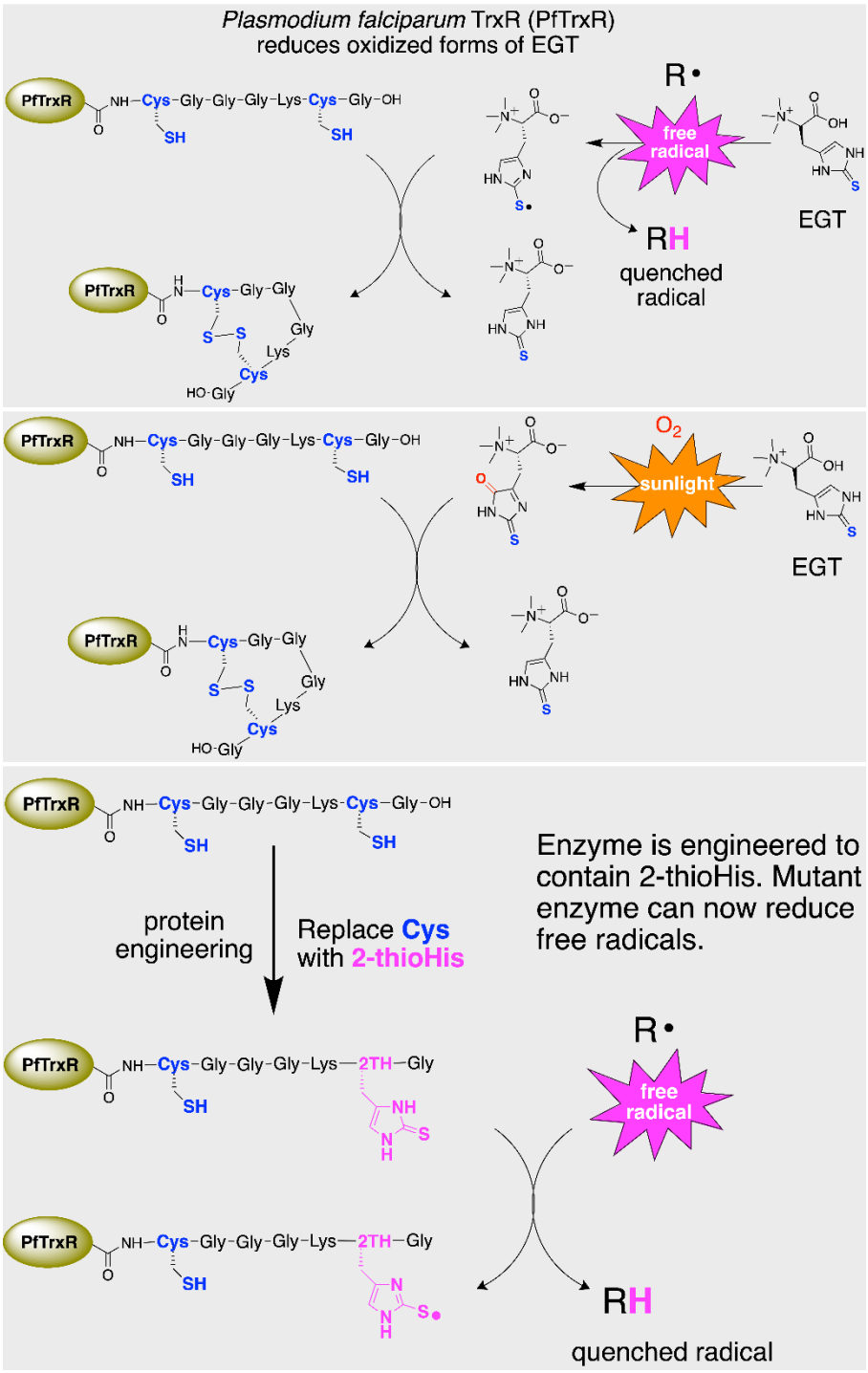

Figure 3: Capture of antioxidant properties of EGT in a mutant TrxR. EGT reduces a radical and becomes oxidized. The wild type PfTrxR reduces the EGT radical (*top panel*), or EGT reacts with $^1O_2$ and is oxidized to 5-oxo-EGT, which in turn can be reduced by PfTrxR (*middle panel*). Here, EGT and PfTrxR work synergistically, but **separately**, to reduce the radical and form a catalytic system. Our idea is to incorporate 2-thioHis into the enzyme directly (*bottom panel)* so that the mutant has a gain of function such that the **mutant enzyme can directly reduce** the free radical since the mutant contains 2-thioHis, an analogue of EGT. Similarly, we believe our mutant could reduce 5-oxo-EGT.

Previous work in the Hondal lab investigated the activity of a different amino acid, selenocysteine, in TrxR enzymes from different species, including *Mus musculus*, *Drosophila melanogaster*, and *Plasmodium falciparum*. The enzymes are produced through recombinant

DNA technology, where a bacterial DNA plasmid has been created to contain the gene that codes for the enzyme from the original organism. This plasmid is then inserted into *Escherichia coli*, and the bacteria uses the plasmid DNA to produce the protein. In the previous study, mutant *Plasmodium falciparum* TrxR enzymes were found to have much higher activity than those of the other species in various biochemical activity assays[13]. For this reason, the *Plasmodium falciparum* enzyme was chosen as the enzyme to be mutated in this study, to see if the introduction of 2-thiohistidine could increase its reactivity even more.

As stated previously, the Hondal group showed that thioredoxin reductase enzymes reduce oxidized forms of EGT. The oxidized form of EGT formed by $^1O_2$, 5-oxo-EGT, was shown to be able to be reduced directly by TrxR. This enzymatic regeneration of EGT through TrxR was the first to be published for EGT[16]. There has yet to be any investigation of the gain in function conferred by a 2-thiohistidine residue inserted in a semisynthetic TrxR enzyme, which is the aim of this project.

As shown in **Figure 3**, we want to create mutant enzymes that contain 2-thiohistidine that take advantage of the unique antioxidant reactions catalyzed by EGT as an individual molecule. Incorporation of 2-thiohistidine into PfTrxR will allow for the mutant to catalyze these reactions directly. This is done through the reaction of 2TH with free radicals, and the enzyme reducing this oxidized 2-TH back to its active form. With this in mind, we created mutant PfTrxR enzymes via semisynthesis to incorporate 2-TH into PfTrxR as shown in **Figure 4**. The process uses an intein that is inserted at the C-terminus of the recombinant protein along with the affinity tag called the chitin-binding domain (CBD) to create a fusion protein. An intein is a type of mobile genetic element that inserts itself into an essential host protein such as a polymerase. The intein catalyzes a self-splicing reaction that removes itself from the host protein and reconstitutes the

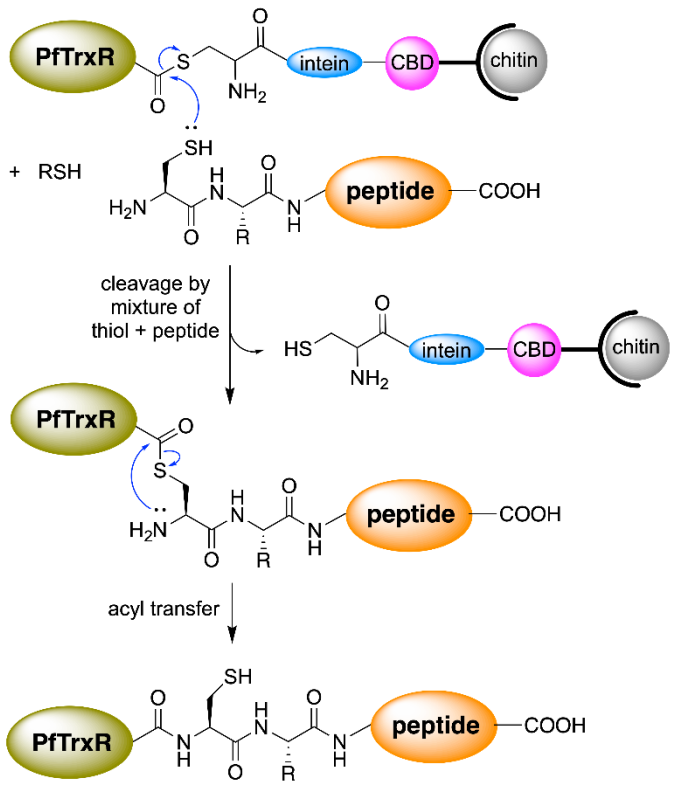

Figure 4: Creation of PfTrxR variants by protein semisynthesis. I used a protein engineering technique called protein semisynthesis that was developed by Xu and coworkers at New England Biolabs[17]. This technique is related to "native chemical ligation developed by Kent and coworkers[18]. Inteins are a type of genetic mobile element. The DNA of the intein inserts itself into the middle of an essential host protein (such as a polymerase) and then catalyzes a splicing reaction so that the host protein is reconstituted. Biotechnology has taken advantage of this for the type of protein engineering shown here. We fused the gene for PfTrxR (missing the C-termiinal 7 amino acids) next to an intein-chitin binding domain (CBD). We then produce the protein in E. coli cells and isolate the fusion protein by affinity chromatography using the chitin tag.

original host protein by joining together of "exteins"[19]. Protein engineering/biotechnology makes use of inteins as shown in **Figure 4** to allow for protein semisynthesis. This enables non-standard amino acids to be incorporated into a protein.

The process of the excision of the intein/CBD is catalyzed by the addition of a thiol-containing compound, in this study *N*-(methyl)mercaptoacetamide (NMA). This creates a thioester at the C-terminus of the protein. The peptide added during the cleavage contains a cysteine residue at the N-terminus, which attacks the thioester on the recombinant protein[17, 18]. Through a rapid rearrangement this ultimately results in the formation of a new peptide bond and the complete semisynthetic protein (**Figure 4**). This allows us to incorporate any amino acid into the C-terminus of the protein as long as it can be put in the synthetic peptide. The chitin-binding domain that is added at the end of the fusion protein allows for easy purification of the fusion protein by use of affinity chromatography.

Protein semisynthesis in this study is made up of three main steps: (i) the synthesis and purification of a large recombinant fragment of the protein in *E. coli* cells which contains a chitin resin affinity tag at the desired place of peptide ligation, in our case the C-terminus. (ii) The synthesis of the desired peptide to be ligated to the recombinant fragment. (iii) Ligation of the desired peptide to the recombinant protein fragment.

Mutant 1 was created to test the hypothesis that the mutant will be able to quench a free radical through the transfer of 1-electron. This is the basic idea stated previously, where the 2TH in the enzyme becomes oxidized, and the enzyme reduces it back to an active form. This was measured by the mutant's ability to metabolize hydroxyl radical. However, various other substrates, including hydrogen peroxide, and hypochlorous acid, which are relevant species in cellular oxidative stress, were also tested for activity.

Mutant 2 was created to test the hypothesis that the mutant will gain the ability to metabolize $^1O_2$ in solution. It was hypothesized that the two 2TH residues would become oxidized by $^1O_2$ and form a disulfide, which would then be reduced by the enzyme back to the active state. This was tested indirectly through the enzyme's consumption of the coenzyme NADPH in the presence of singlet oxygen.

Mutant 3 will test the hypothesis that the 2TH residue will protect another active amino acid residue on the enzyme from oxidation. This is an extension of the previous hypothesis. We have previously created a mutant of PfTrxR that contains selenocysteine (Sec) and this mutant reacts with various substrates[20]. Singlet oxygen likely inactivates the redox active Sec through oxidation of the selenium atom present in this residue. We hypothesize that addition of 2TH will protect the Sec residue so that the mutant enzyme will remain active in activity assays after exposure to $^1O_2$. **Figure 5** summarizes these hypotheses.

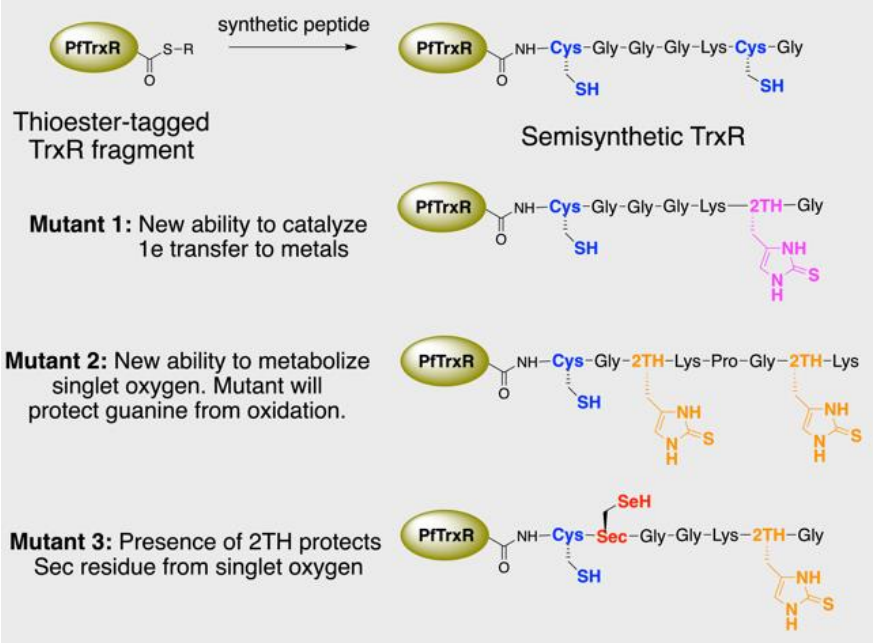

Figure 5: Image showing process of mutant peptide ligation to the recombinant PfTrxR fragment. Mutant 1 containing one 2-thiohistidine residue is expected to react with metal free radical species. **Mutant 2** is expected to gain the ability to reduce singlet oxygen. **Mutant 3** contains one 2-thiohistidine residue which is expected to protect the Sec residue from oxidation via singlet oxygen, therefore allowing the enzyme to retain activity.

Significance:

Although EGT has been shown to individually quench singlet oxygen, it has yet to be inserted into an enzyme as a possible active residue. In conjunction with TrxR, the proposed catalytic antioxidant mechanism would be the first of its kind. If the enzyme is able to efficiently reduce the oxidized 2TH residues, this would mean that mutant TrxR enzymes containing 2TH could be a very efficient cytoprotectant. For example, an enzyme which can quench $^1O_2$ faster or better than other antioxidant molecules would greatly reduce the production of 8-oxoguanine, preventing cellular damage.

The prevention of the oxidation of guanine would be biologically relevant, since there are often high levels of this species found in patients with Alzheimer's disease, Parkinson's disease, and Huntington's disease. These diseases are often characterized by high levels of oxidative stress due to reactive oxygen species, including $^1O_2$[21]. If the mutant protein produced in this

project is found to greatly reduce guanine oxidation *in vitro*, this could open up further study into its use as a preventative measure for oxidative damage in the body.

Methods:

Production of Semisynthetic PfTrxR Enzymes:

All C-terminal peptides used in creating the mutant PfTrxR enzymes were created using solid-phase peptide synthesis as described in previous published work from the Hondal group[22]. Selenocysteine-containing peptides had to be deprotected after synthesis due to the presence of the 4-methoxybonzoyl (Mob) protecting group on the selenocysteine residue. They were deprotected using DTNP, in a ratio of 2 equivalents of DTNP to 1 equivalent of the peptide. The dry peptide, DTNP, and 1.96 mL cleavage cocktail of 96:2:2 trifluoroacetic acid (TFA):triisopropylsilane (TIS):water were combined and incubated for 1.5 hours, then precipitated into cold ether. Several ether washes were performed and then the pellet was dried and lyophilized. Direct-inject mass spectroscopy was used to confirm the sequence and identity of the peptides before their use in creating the semisynthetic protein.

In order to produce the recombinant PfTrxR fragment, plasmid DNA was inserted into *E. coli* cells. The recombinant plasmid contains the PfTrxR gene and the gene for an affinity tag, chitin, used in protein purification. The recombinant PfTrR fragment was produced in *E. coli* cells (cell line C2566). Cells were transformed utilizing heat shock for 45 seconds at 42 ℃ and placed back on ice for 10 minutes. Successfully transformed cells were selected for after plating on LB-agar plates with 100 μg/mL ampicillin as a selection marker, since a gene for ampicillin resistance was present in the plasmid DNA. The cells were grown in a 2 mL LB starter culture for 24 hrs at 37 ℃. Starter culture was then added to one liter of TB media with 100 μg/mL ampicillin at 37 ℃

and allowed to grow to an OD of 1before harvesting. Cells were harvested by 10-minute centrifugation at 10,000 rpm. Pellets were then frozen at -20 ℃.

To harvest the recombinant PfTrxR fragment from the cell pellets, they were first thawed and homogenized in chitin "A" buffer (50 mM MOPS, 150 mM NaCl, pH 7). The cells were then lysed via sonication. The homogenate was centrifuged for 40 minutes at 10,000 rpm, or until the supernatant was no longer cloudy. The supernatant was loaded onto a chitin affinity column (NEB) equilibrated with chitin "A" buffer. The column was then washed with chitin "A" until the A_{280} of the effluent was approximately 0.1. Next, the column was washed with chitin "B" buffer (50 mM MOPS, 500 mM NaCl, pH 7) until the A_{280} of the effluent was below 0.1.

After washing the chitin resin was then saved for the cleavage and ligation process to form the complete active enzyme. The cleavage/ligation reaction contained approximately 25 mL loaded chitin resin, 25 mL 120 mM *N*-(methyl)mercaptoacetamide (NMA) in chitin "B" buffer with an adjusted pH of 8.5. The reaction was allowed to proceed overnight in the dark at room temp. To collect cleaved protein the resin was drained using the column frit, and the flowthrough washed and concentrated by centrifugation in 30,000 MWCO concentrators with S200 buffer (50 mM potassium phosphate, 500 mM NaCl, 1 mM EDTA, pH 8.0). The concentration of enzyme was determined using the absorbance at 460 nm (absorbance of FAD coenzyme) and Beer's Law.

Characterization of Enzymatic Activities:

In order to investigate the activity of the enzymes a variety of common activity assays were performed. These included thioredoxin (Trx), hydrogen peroxide (H_2O_2), Ellman's Reagent (DTNB), and hypochlorous acid (HOCl). These assays were also used to characterize the other mutant enzymes. All assay reactions were carried out in 100 µM potassium phosphate, 1mM EDTA, pH 7 buffer and contained 200 µM NADPH.

Thioredoxin is the natural substrate of the wild-type enzyme. This was used to ensure that the creation of the enzyme was successful. The concentration of Trx was varied from 10 to 150 µM in a minimum of six points to generate a Michaelis-Menten curve. Enzyme concentrations varied for each enzyme from 10- 45 nM in order to get sufficient activity above baseline to generate a curve, was maintained for the whole assay, and accounted for in the Michaelis-Menten analysis, allowing for comparison between mutants. Activity is monitored through the consumption of NADPH at 340 nm.

Ellman's reagent is a disulfide compound. Here it is used to investigate the enzyme's activity and compare between mutants. DTNB concentration was varied from 200 to 750 µM in each assay to include a minimum of six data points. Enzyme concentrations varied from 2 to 20 nM, again to get sufficient activity above baseline to generate the Michaelis Menten curve. These concentrations were accounted for when plotting the data, allowing for comparison between mutants. Activity was monitored through the production of TNB at 412 nm.

Hydrogen peroxide (H_2O_2) is a common species used for investigation of enzymatic activity with reactive oxygen species. Hydrogen peroxide concentration was varied from 2.5 to 50 mM in each assay. Enzyme concentrations for Mutant 3 and the serine containing analogue of Mutant 3 were 30 nM. The other mutant enzymes and wild-type enzyme did not have sufficient activity with H_2O_2 to produce a Michaelis-Menten curve. Activity was monitored through the consumption of NADPH at 340 nm.

The hypochlorous acid assay was performed with all mutants and the truncated enzyme. At the time this assay was performed the wild-type enzyme was not available. HOCl concentration was varied from 3 to 30 mM. The enzyme concentration was 20 nM for all enzymes each individual assay. Activity was monitored through the consumption of NADPH at 340 nm.

Glutathione/Selenite Hydroxyl Radical Assay

In order to generate the hydroxyl radical without the use of a metal, glutathione and selenite were used in aerobic solution. Drawing from work by Dereven'kov et. al[23], and through trial and error, a ratio of GSH: Selenite of 25:1 was determined to be a good ratio to use. An enzyme concentration of 250 nM was used. The concentrations of GSH and selenite in the assay were 250µM and 10µM, respectively. This assay was carried out in 100 µM potassium phosphate 1mM EDTA pH 7 buffer and contained 200 µM NADPH. Selenite was used to initiate the reaction. The reaction was followed for 10 minutes, and NADPH consumption measured through the change in absorbance at 340 nm per minute.

Singlet Oxygen Consumption:

In order to determine if mutant 2, as well as the other mutants, gained the ability to metabolize singlet oxygen, a new assay was established. This utilized two control reactions and one experimental reaction to determine whether the enzyme was consuming NADPH in the presence of singlet oxygen. The reactions were as follows:

1. Control: *NADPH, Rose Bengal, D_2O*: Shows how much NADPH is consumed by singlet oxygen generation alone. Label: NO enzyme.
2. Control: *NADPH, Enzyme, D_2O:* Shows how much NADPH is consumed by enzyme alone. Label: NO $^1O_2$.
3. Experimental: *NADPH, Enzyme, Rose Bengal, D_2O:* Shows whether the enzyme is turning over singlet oxygen when reactions 1 + 2 are used as controls.

Reactions had a total volume of 100 µL. The reaction mixture was 90% D_2O, 800 µM NADPH, 300 nM enzyme, 10 µM Rose Bengal, and brought to 100 µL with 100 µM potassium

phosphate/1 mM ETDA, pH 7 buffer. Rose Bengal was added to the mixture last. The reaction was mixed, and a sample taken and diluted 5X in assay buffer to measure initial absorbance at 340 nM. The remaining mixture was exposed to a halogen bulb (Philips 415729 500-Watt 4.7-Inch T3 RSC 120-Volt Light Bulb with double ended base) in a 4 ℃ circulating water bath for 5 minutes. After incubation under light another sample was taken and diluted 5X to measure final absorbance at 340 nm. This procedure was repeated for each reaction with each mutant in triplicate. Using the change in absorbance at 340 nm and Beer's law, the percent of NADPH consumed was determined for each reaction.

Singlet Oxygen Inactivation:

For this assay, we used a mutant of PfTrxR that contains a selenocysteine residue but no 2-thiohistidine as a control. This mutant reduces Trx as well as other small molecules. When this enzyme is exposed to singlet oxygen, the enzyme is inactivated. The new mutant shown in **Figure 5** has selenocysteine and 2-thiohistidine. This mutant is exposed to singlet oxygen by addition of rose bengal, D_2O, and light, and then its ability to reduce Trx is measured through monitoring NADPH consumption at 340 nm.

A similar procedure to that described in the singlet oxygen metabolism assay is followed, with the exception of the initial and final A_{340} readings and the four reactions as listed below:

1. Control: *Enzyme, Rose Bengal, D_2O.*
2. Control: *NADPH, Enzyme, D_2O. (Treated as 100% baseline activity)*
3. Control: *NADPH, Enzyme, Rose Bengal, D_2O in* ***Dark.***
4. Experimental: *NADPH, Enzyme, Rose Bengal, D_2O.*

After a 5-minute incubation under light or dark the reaction mixture is diluted into the cuvette, so the enzyme concentration is 10 nM in the assay, and NADPH is added to the assay to a concentration of 200 µM as in previous activity assays. The assay is initiated through addition of Trx to a concentration of 120 µM, and activity monitored through NADPH consumption by monitoring the change in absorbance at 340 nm.

In order to determine if the 2-thiohistidine is a better protector of the selenocysteine residue when in the primary structure of the protein itself, rather than endogenous 2-thiohistidine in solution, the same experiment described previously was performed with the control enzyme with and without 0.5 µM, 10 µM, and 1 mM 2-thiohistidine in the reaction mixture incubated under light. After incubation with $^1O_2$, the reactions were subjected to ultrafiltration to remove the 2-thiohistidine from solution, as it can react with NADPH in solution, and artificially raise activity. It was assayed with 120 µM Trx in the same fashion as in the previous method, excepting an enzyme concentration of approximately 30 nM.

Analyzing Assay Data

In order to analyze the assay data to determine the activity of the mutant enzymes, standard analysis of Michaelis-Menten kinetics plots generated by GraphPad™ was used. From these graphs, the maximum velocity of the reactions and catalytic efficiency of each enzyme will be determined in their respective assays for further comparison. All assay data was normalized for enzyme concentration when curves were generated as to allow for easier comparison between mutants' activity.

Results:

Characterization of Enzymes:

All mutants and the wild-type enzyme were characterized with general enzymatic assays including Trx, H_2O_2, DTNB, and HOCl. Only the selenocysteine-containing mutants had significant activity with H_2O_2, and the 2TH containing mutant had slightly higher activity than the serine containing mutant, although they were similar. The selenocysteine-containing mutants also had slightly higher activity in the other assays. The serine-containing mutant had very high activity with DTNB. Overall, the other mutants had similar activities with Trx and DTNB **(Table 1)**. Michaelis-Menten curves for all data in **Table 1** can be found in **Supplemental figures 1-6**. Normalized velocity as seen on the y-axis of all curves is the moles substrate/min/mole enzyme, as this velocity is "normalized" for the differing concentrations of enzyme in each assay.

Table 1: Catalytic efficiencies of mutant and wild type enzymes. All values came from Michaelis-Menten curves of each assay with values normalized for enzyme concentration. See curves in supplemental materials. N/C is not completed.

Sequence of C-terminal peptide	**Enzyme #**	**Trx ($min^{-1}M^{-1}$)**	**H_2O_2 ($min^{-1}M^{-1}$)**	**DTNB ($min^{-1}M^{-1}$)**
CGGGK**2TH**G	1	7.37×10^4	N/A	6.38×10^5
CG2THKPG**2TH**K	2	N/C	N/A	8.61×10^5
CUGGK**2TH**G	3	3.05×10^7	1.79×10^4	1.79×10^6
CUGGKSG	4	1.83×10^7	1.54×10^4	1.43×10^8
CGGGKCG (wild type)	5	8.90×10^6	N/A	N/C
PfTrxRΔ7	6	N/A	N/A	5.06×10^5

In the HOCl assay, mutant 2, with two 2TH residues, had the highest activity compared to the truncated and selenium containing mutant. Mutant 1, with one 2TH residue, had similar activity to mutant 2 at the 20 mM HOCl point, but had lower activity previous to that point. All enzymes lost all activity at 30 mM HOCl **(Figure 6)**.

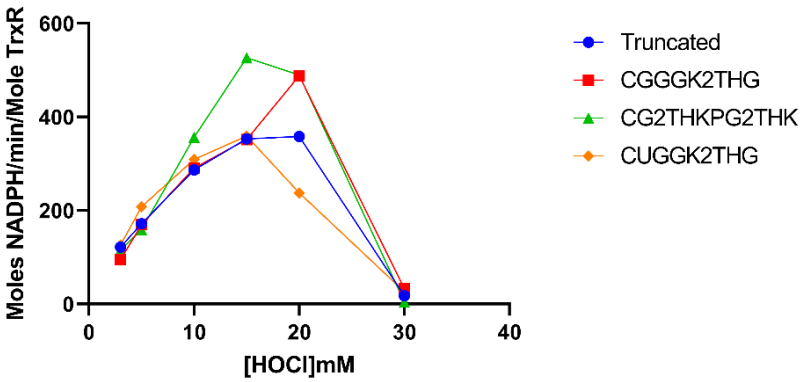

Figure 6: Hypochlorous acid assays of mutants 1-3 and the truncated enzyme. Normalized for enzyme concentration.

Hydroxyl Radical Assay:

Assays done with the GSH/selenite hydroxyl radical generation system were not conducive to Michaelis-Menten kinetics. A lag phase was observed for each reaction. The reactions were followed for 10 minutes, and a jump in activity was detected at 6 minutes with the truncated enzyme, and 4 with the mutant. The slope at these points were -0.0608 and -0.0861, respectively **(Figure 7)**.

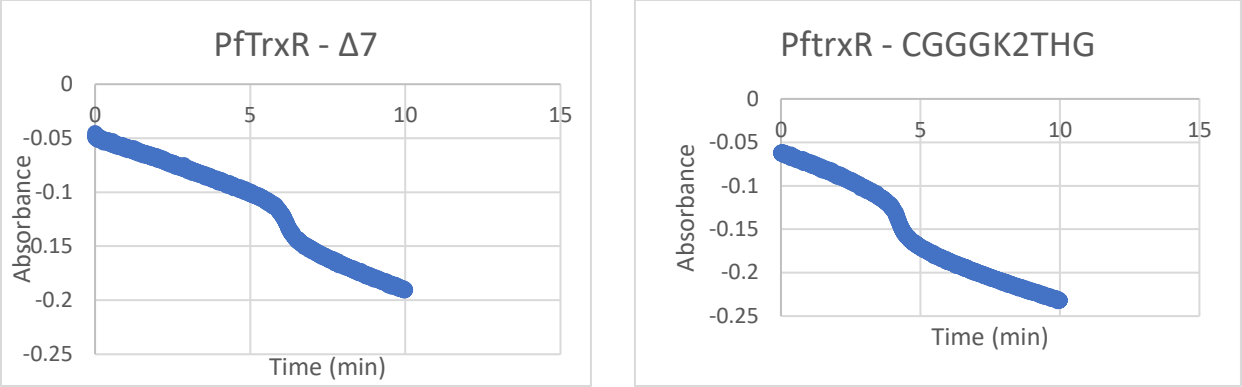

Figure 7: Glutathione/selenite hydroxyl radical assay. Truncated enzyme (Δ7) and Mutant 1 assayed with 25:1 ratio of glutathione: selenite and the change in absorbance at 340 nm followed for 10 minutes. Maximum slope for truncated enzyme: -0.0608 at 6 minutes. Maximum slope for Mutant 1: -0.0861 at 4 minutes.

Singlet Oxygen Metabolism:

Only Mutant 3, containing selenocysteine and 2TH, was found to have increased NADPH consumption, and therefore $^1O_2$ consumption in solution. It's corrected value of % NADPH consumption was +6.9, which was higher than other values. Mutant 1 had a corrected value of +2.5, the only other positive value for % NADPH consumed. Mutant 2, with two 2TH residues, had a corrected value of -2.4. The serine containing mutant also had a negative value of -3.1. Interestingly, the wild-type enzyme had the most negative value, -14.1. Standard deviations varied between mutants.

Table 2: Singlet Oxygen metabolism via NADPH consumption. Percent NADPH consumption values as calculated from absorbance values at 340nm taken before and after exposure to light/singlet oxygen, as well as corrected values. Corrected values are the result of the % NADPH consumed by singlet oxygen alone subtracted from % NADPH consumed by singlet oxygen and the enzyme, giving us net NADPH consumed by the enzyme.

Mutant	**PfTrxR – CGGGK2THG**	**PfTrxR – CG2THKPG2THK**	**PfTrxR – CUGGK2THG**	**PfTrxR - CUGGKSG**	**PfTrxR - Δ7**	**PfTrxR - WT**
% NADPH consumed by $^1O_2$	45 (±4.79)	45 (±4.79)	45 (±4.79)	45 (±4.79)	45 (±4.79)	45 (±4.79)
% NADPH consumed by Enzyme (No $^1O_2$)	6.3 (±2.25)	6 (±1.98)	3.2 (±2.55)	1.9 (±1.28)	6.2 (±3.21)	3.3 (±1.70)
% NADPH Consumed by $^1O_2$ and Enzyme	47.5* (± 9.02)	42.6 (± 4.08)	51.9 (± 2.56)	41.9 (±3.45)	42 (±8.08)	30.9 (±5.03)
Δ[a]	+2.5 (± 9.02)	-2.4 (± 4.08)	+6.9 (± 2.56)	-3.1 (±3.45)	-3 (±8.08)	-14.1 (±5.03)

Singlet Oxygen Inactivation:

As seen in **Table 3**, Mutant 3 retained almost 100% of activity with Trx when exposed to singlet oxygen in solution. The control mutant, containing serine, retained only 32.2% of activity and the wild type 44.2% of activity. When in solution with singlet oxygen without NADPH all lost more than 50% activity. 100% activity was determined to be exposure of the reaction to light without singlet oxygen.

Table 3: Singlet oxygen inactivation assay. Activity referred to is enzymatic activity in Trx assay with 120 µM Trx and 10 nM enzyme as monitored by A340. Values given in average slopes as well as % retained activity. 100% activity assigned to reaction exposed to light but without singlet oxygen generation.

Enzyme #	**3**	**4**	**5**
Reaction	**PfTR – CUGGK2THG** ΔA340 (% of control)	**PfTR – CUGGKSG** ΔA340 (% of control)	**PfTR – WT** ΔA340 (% of control)
Rose Bengal and Light **+NADPH**	0.0216 (99.1%)	0.0185 (32.2%)	0.0099 (44.2%)
Rose Bengal and Light. **–NADPH**	0.0046 (21.1%)	0.0088 (15.3%)	0.0081 (36.2%)
Rose Bengal and Dark	0.0242 (111%)	0.0591 (102%)	0.0216 (96.4%)
Light w/o Rose Bengal (control)	0.0218 (100%)	0.0575 (100%)	0.0224 (100%)

When the serine mutant was incubated with varying concentrations of exogenous 2thioHis, it was determined that 1mM 2TH was required to retain 77% of enzymatic activity (**Table 4**). Increased 2TH concentration would be necessary to protect 100% of activity. Exact concentration of enzyme is not known in these assays, due to the process of washing required to remove excess 2TH from the solution before assaying with Trx as to prevent artificially high activity.

Table 4: Enzymatic activity protection via exogenous 2TH. Retained enzymatic activities after exposure to singlet oxygen with varying concentrations of exogenous 2TH. The assay contained 120 µM Trx, and approximately 30 nM enzyme to account for loss in the washing process.

[2TH] in Reaction	**% Retained Activity**
0.5 µM	22.6
10 µM	27.3
1 mM	77

Discussion:

Creation and Characterization of Enzymes:

All the mutant enzymes and the wild-type enzyme were created successfully through protein semi-synthesis. Mass spectrometry showed that each of the C-terminal peptides were made successfully through solid-phase peptide synthesis previous to ligation to the recombinant fragment of protein. The mass spectra can be found in **Supplemental Figures 7-11**. In the process of cleavage and ligation of the mutants, protein aggregate was seen. This was not seen in the cleavage and ligation of the wild-type enzyme. This may have been due to a multitude of reasons, one of which being that the cleavage conditions were not perfectly optimized to the protein, such as pH, concentration of NMA, or temperature, causing denaturation and resulting in precipitation of some of the protein out of solution. However, due to the fact that this did not occur in the case of the wild-type enzyme, it is possible that the mutant C-terminal peptides did not adequately stabilize the protein upon ligation as the native peptide did, resulting in the loss of protein. Overall, despite the loss of protein in this step, active, correct mutant enzymes were created.

Mutant Enzymatic Activities:

In order to characterize the many enzymes for their level of activity and to determine whether there may have been a gain in activity with the addition of 2TH in the different configurations, different assays were performed. Trx and DTNB assays were done to ensure that each of the mutants had canonical activity with disulfide species, their natural substrate. All of the enzymes had similar activity in these assays, with the selenocysteine containing mutants having slightly higher activity as seen in **Table 1**. This was not unexpected, as selenocysteine containing TrxR enzymes have been seen to have high activity[13]. One unexpected result was the very high activity of the serine mutant (Enzyme #4) with DTNB. This enzyme had a catalytic efficiency of

1.43 x 10^8 $min^{-1}M^{-1}$ with DTNB. Since this enzyme does not have a sulfur containing residue, such as 2TH or cysteine, where the serine is, we did not expect it to have increased activity with a disulfide-containing substrate.

The selenocysteine containing mutants were also the only ones observed to have some activity in the H_2O_2 assay. This assay was done because H_2O_2 is a common reactive oxygen species in cells, however wild-type TrxR does not metabolize it well, so we wanted to investigate whether our mutant enzymes would gain the ability to turn catalyze the reduction of H_2O_2. Although there was some activity with H_2O_2 in our mutants, it did not stand out as a main activity of the enzymes, with catalytic efficiencies of 1.79 x 10^4 and 1.54 x 10^4 $min^{-1}M^{-1}$.

The final assay done to characterize the enzymes was a HOCl assay. This was done because HOCl is another common chemical species associated with reactive oxygen species and oxidative stress in cellular environments, as it is naturally produced by peroxidase enzymes and used in our immune response[24]. The mutant enzymes had some activity with HOCl, but unfortunately the data did not fit the Michaelis-Menten analysis. However, looking at **Figure 6**, the enzyme with the highest activity overall was Mutant 2, containing two 2TH residues. This may show a new activity in this enzyme due to the introduction of two 2TH residues becoming oxidized into a disulfide and then catalytically reduced by the enzyme. The lowest activity was seen in the truncated form of the enzyme and Mutant 3, the enzyme with selenocysteine and 2-TH. This opposes the previous assays, where the selenocysteine containing enzymes had higher activity. This may be due to a multitude of reasons, but it is possible that the HOCl is affecting the selenocysteine residue and leading to some inactivation of the enzyme. Overall, more study is needed to elucidate the results of this data.

Hydroxyl Radical Assay:

Mutant #1 was hypothesized to gain the ability to catalyze 1-electron reductions and gain reactivity with various substrates, including the hydroxyl radical. In order to determine its reactivity with the hydroxyl radical, a procedure was needed to generate the hydroxyl radical without the use of a metal, such as is used in the Fenton reaction. This is because metals have reactivity with NADPH in solution, and this would result in artificially higher activity in the assay since NADPH is a required coenzyme for the reaction. Using the truncated enzyme as a control, as the wild-type enzyme was not available at the time of this experiment, it was observed that there was a lag phase in the assay. The truncated enzyme had a burst of activity at 6 minutes, while the mutant had a burst at 4 minutes. The slopes at these points were -0.0608 and -0.0861, respectively **(Figure 7)**. The difference in timing of these bursts in activity as well as the higher slope seen with the mutant may be indicative of increased activity that the 2TH is conferring to the mutant enzyme. However, since the truncated enzyme is missing the entire C-terminal active site, it would be better to compare the wild-type enzyme in this experiment. If there were more time to continue this project, this would be an avenue of further study with the wild-type enzyme as well as the other mutants.

Singlet Oxygen Metabolism:

Using the singlet oxygen consumption/NADPH consumption assay, Mutant #3 was the only mutant observed to have increased metabolism of $^1O_2$. This is because it was consuming NADPH on top of what singlet oxygen was consuming on its own, observed in its corrected value of NADPH consumption of +6.9%. Additionally, its standard deviation of $\pm$2.56 is not so large as to potentially result in a negative number. This was not the case with Mutant #1, as seen in **Table 2**. Despite the +2.5% corrected NADPH consumption value, the standard deviation for this point

was ±9.02, making this data less trustworthy. Overall, more replicates would help to verify this data. With more time, this would be a future path for this study. Finally, the wild-type enzyme had a very negative value, -14.1%, for a corrected NADPH consumption value. This means that less NADPH was being consumed with enzyme present than with just $^1O_2$. Although other enzymes had negative values, they were much closer to zero and within range of error. Although we are unsure of why the wild-type had this effect in solution, it may be that it is acting to "protect" NADPH in solution through singlet oxygen preferentially oxidizing the enzyme over NADPH. Alternatively, this may mean that this *Plasmodium Falciparum* TrxR may have its own unique activity with $^1O_2$ that differs from TrxR enzymes found in other species.

Although it was originally hypothesized that Mutant #2 would gain the ability to metabolize $^1O_2$, Mutant #3 was the enzyme observed to gain some activity to metabolize $^1O_2$ in this experiment. This goes along with increased activity of Mutant #3 in other assays, despite the phenomenon of selenocysteine is likely inactivated by $^1O_2$. This is addressed by the $^1O_2$ inactivation assay, but ultimately, we see that the 2TH residue in this enzyme protects the selenocysteine residue, which likely also plays a part in this enzyme's observed metabolism of singlet oxygen in this experiment. From this data, the mechanism as seen in **Figure 8** is proposed to explain the metabolism of $^1O_2$ by this enzyme. In the future this assay could be further developed to more accurately represent physiological conditions with the amount of $^1O_2$ monitored in solution so as not to overproduce and consume significant amounts of NADPH or cause significant damage to the enzymes themselves through side reactions. Additionally, it is possible that the current configuration of the C-terminus of this active site is not optimized for the metabolism of $^1O_2$, and further study could be done in modeling a peptide which would allow for optimal enzymatic activity in reducing the 2TH residue. Finally, multiple replicates are imperative to acquire

trustworthy data, and in future studies replicates exceeding triplicate should be done as time allows.

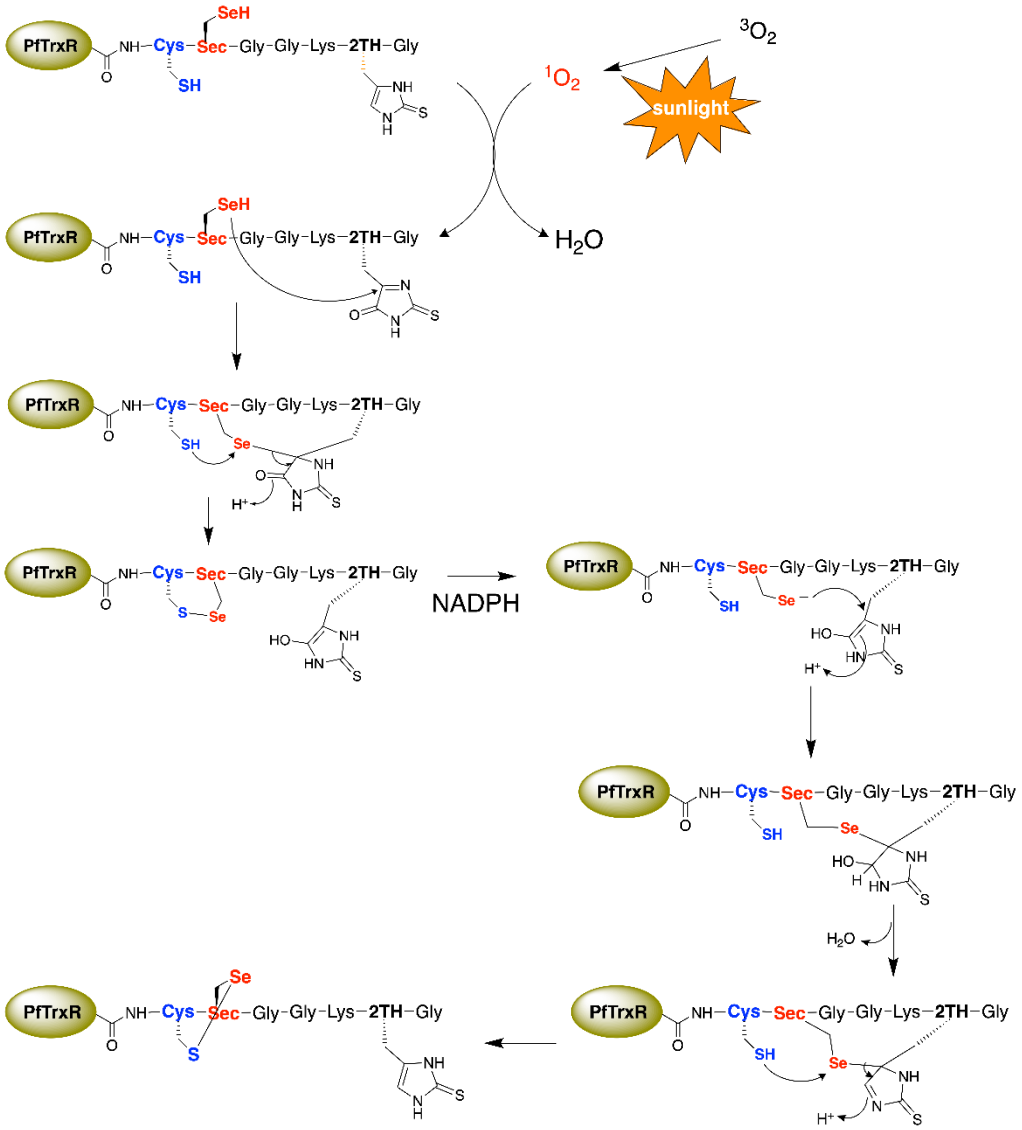

Figure 8: Potential mechanism of singlet oxygen catalysis by mutant 3. Proposed mechanism of activity of the third mutant with singlet oxygen begins with oxidation of the 2-thioHis residue. Following this oxidation, the selenium of the Sec residue and sulfur of Cys reduce the 2-thioHis, becoming oxidized and forming a selenosulfide. The enzyme would then be able to be reduced back to its active state, just as occurs with WT enzyme when the C-terminal disulfide is reduced. This mechanism mimics the mechanism proposed by Oumari et al[10], except the enzyme uses a Cys-Sec dyad in place of glutathione.

Protection from Singlet Oxygen Inactivation:

Mutant #3 was created to investigate how a 2TH residue in the C-terminus of the enzyme may protect an active selenocysteine residue. A mutant identical to Mutant #3, excepting a serine in place of the 2TH residue, was created as a control in addition to the wild-type enzyme. When exposed to $^1O_2$ in the presence of NADPH, Mutant #3 was able to retain almost 100% of activity with the natural substrate Trx, in comparison to the serine mutant and wild-type which each retained only 32.2% and 44.2% of activity, respectively **(Table 3)**. This data supported the hypothesis that the 2TH in the C-terminus protected the active selenocysteine residue. In order to further investigate the protective effects of 2TH, it was used exogenously with the serine mutant to determine if it could provide the same protection as the residue inserted in the C-terminal active site.

Oxidized 2TH in solution can be reduced by NADPH, and therefore needed to be washed from the solution before assaying the enzyme to prevent artificially increased activity. The washing process led to some loss of protein and required the addition of more reaction mixture for the activity assay. Due to this the exact concentration of enzyme in the assay was unknown but estimated to be 30 nM. As seen in **Table 4**, 1mM exogenous 2TH was needed for the enzyme to retain 77% activity with Trx. With the mutant, PfTrxR–CUGGK2THG, activity was almost entirely conserved at a concentration of 300 nM (the enzyme concentration) in the initial reaction with $^1O_2$. This is a significantly lower concentration than what was needed with exogenous 2TH and shows the advantage to having 2TH in the primary structure of the protein at the C-terminus, as it is localized to the residue it is protecting and not just free in solution.

Conclusion:

In this project six semi-synthetic PfTrxR enzymes were successfully created, two of which were the wild-type and truncated enzymes. The three mutant enzymes containing 2TH were designed and created to test three hypotheses about the activity that inserting the non-natural amino acid 2TH would confer to the naturally occurring enzyme. First, with Mutant #1, that 2TH replacing the C-terminal active cysteine in its native position would introduce the ability to carry out one-electron reduction reactions. Second, that Mutant #2, containing two 2TH residues would gain the ability to metabolize $^1O_2$. Third, that a 2TH residue in Mutant #3 would protect the activity of a selenocysteine residue from oxidation via $^1O_2$. In testing all three hypotheses, all mutants were used, which enabled us to observe some unexpected results.

In testing hypothesis one, it was determined that Mutant #1 had increased activity with the hydroxyl radical in the GSH/selenite assay in comparison with the truncated enzyme, supporting the hypothesis that inserting 2TH into the C-terminus of the enzyme would confer the ability to catalyze one electron reduction reactions without the use of Trx, its partner disulfide protein. In testing hypothesis two, data did not support that Mutant 2 gained the ability to metabolize $^1O_2$. However, Mutant #3, containing selenocysteine and 2TH, was seen to have increased NADPH consumption in the presence of $^1O_2$, indicating catalytic activity. This would be a gain of function for this enzyme, likely as a result of the addition of the 2TH residue. Finally, hypothesis three was supported by the $^1O_2$ inactivation experiment, where Mutant #3 was able to retain almost 100% of its activity with the substrate Trx after incubation with $^1O_2$ in comparison to the truncated and wild-type enzymes which both lost more than 50% of activity. Overall, in further study additional replicates would strengthen the data collected. Next steps in this project could include beginning studies into the optimization of the C-terminal peptide sequence of Mutant #3, as well as

investigating the protection it could provide to biological molecules such as guanine which have relevance in human health. Finally, it may be interesting to dive deeper into all of the mutants' activity with the hydroxyl radical. Overall, this study was able to utilize the method of protein semisynthesis to successfully create mutant PfTrxR enzymes containing 2TH, and show the resultant changes in enzymatic activity.

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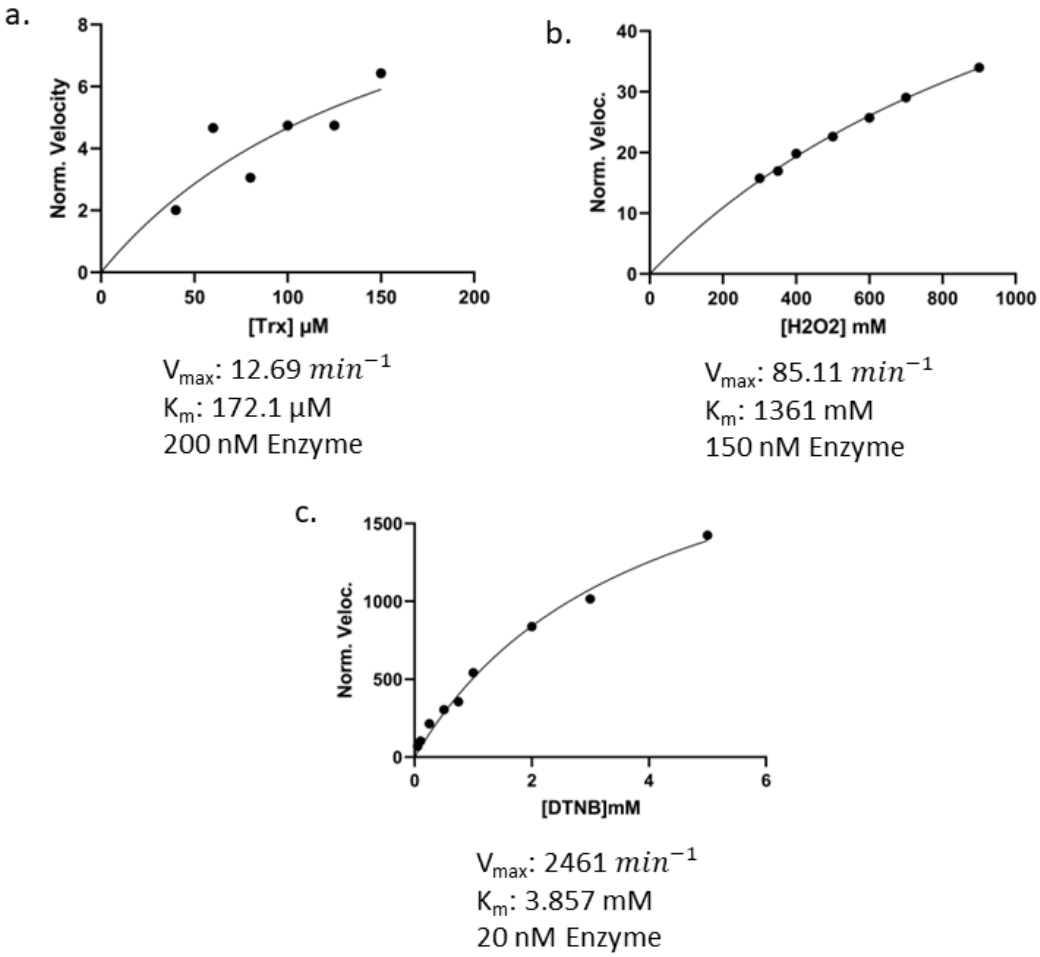
Supplemental Figures

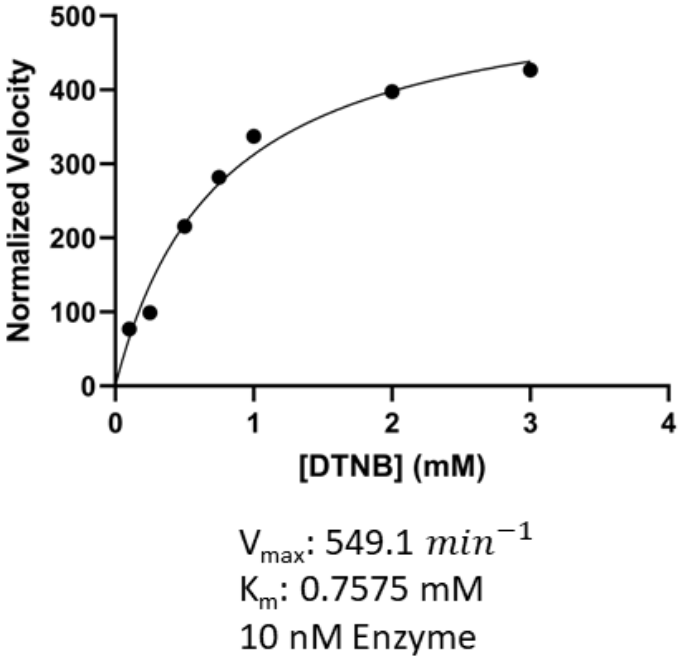


V_{max}: 12.69 min^{-1}
K_m: 172.1 µM
200 nM Enzyme

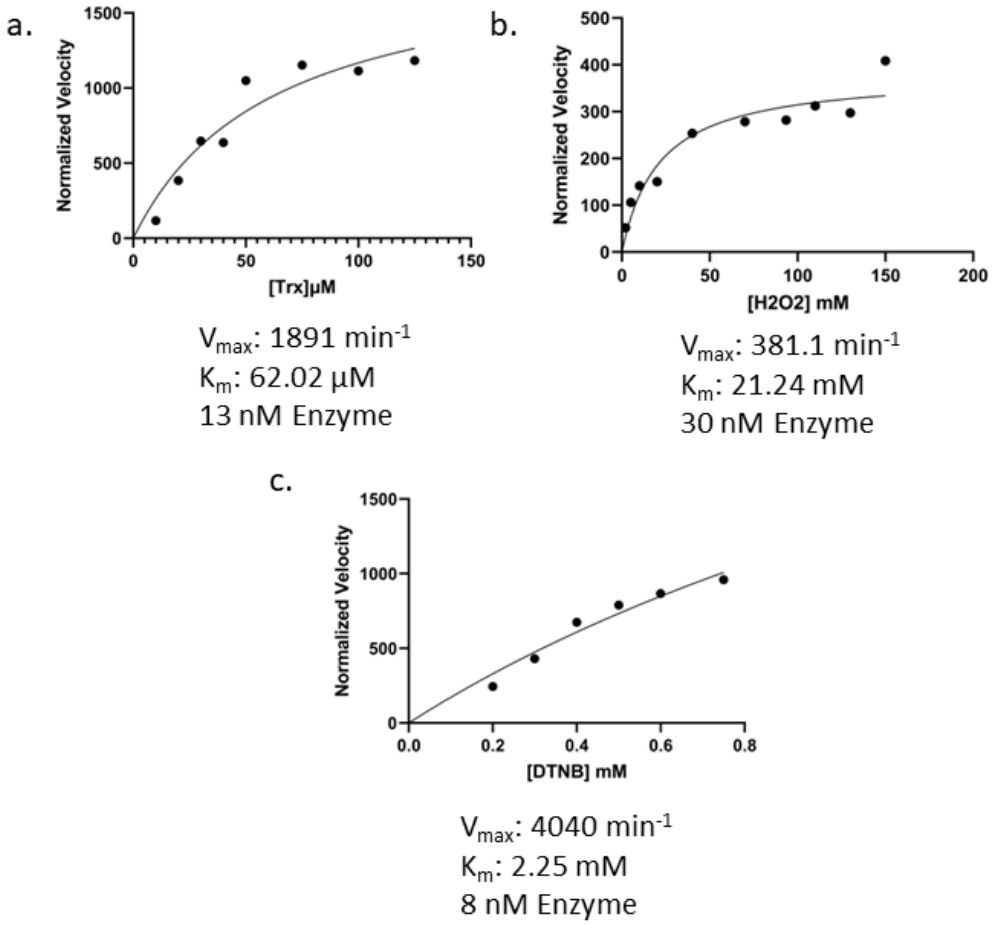
V_{max}: 85.11 min^{-1}
K_m: 1361 mM
150 nM Enzyme

V_{max}: 2461 min^{-1}
K_m: 3.857 mM
20 nM Enzyme

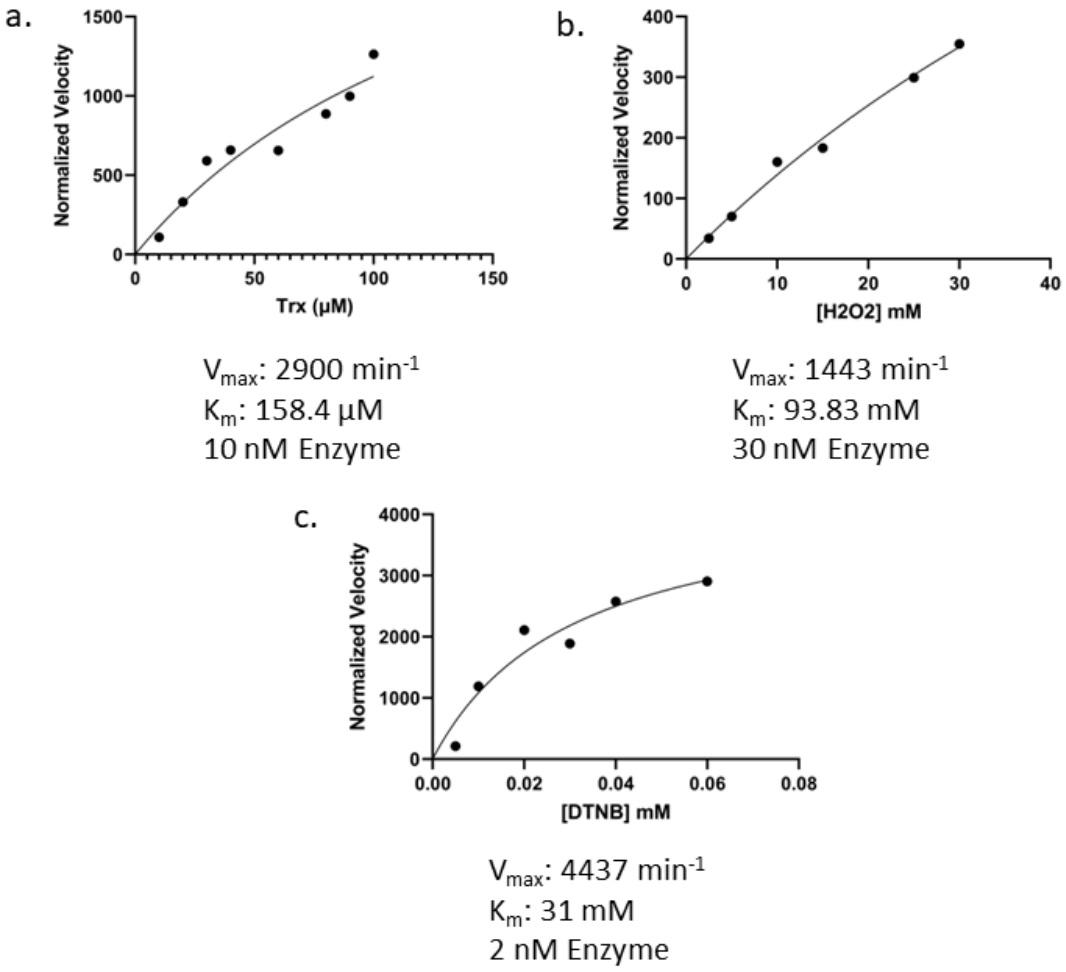
Supp. Figure 1: Michaelis-Menten Curves of Mutant 1 (PfTrxR – CGGGK2THG) Activity assays. (a) Thioredoxin Assay with 200 nM enzyme. Normalized velocity is moles NADPH/min/mole PfTrxR. (b) H_2O_2 assay with 150 nM enzyme. Normalized velocity is moles NADPH/min/mole PfTrxR. (c) Ellman's Reagent (DTNB) Assay with 20 nM enzyme. Normalized velocity is in moles TNB/min/mole PfTrxR.



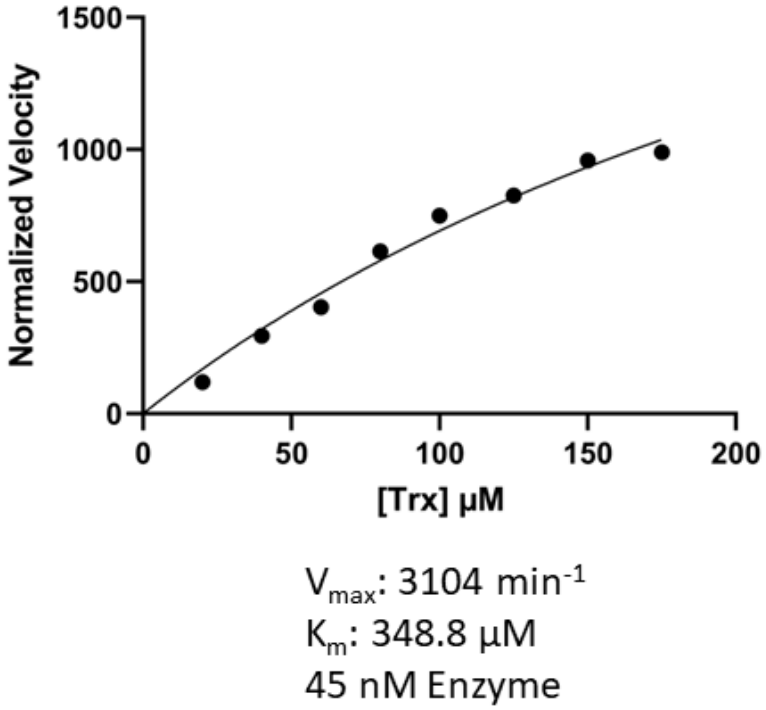
Supp. Figure 2: Michaelis-Menten Curves of Mutant 2 (PfTrxR – CG2THKPG2THK) DTNB Activity Assays. Normalized velocity is moles TNB/min/mole PfTrxR.



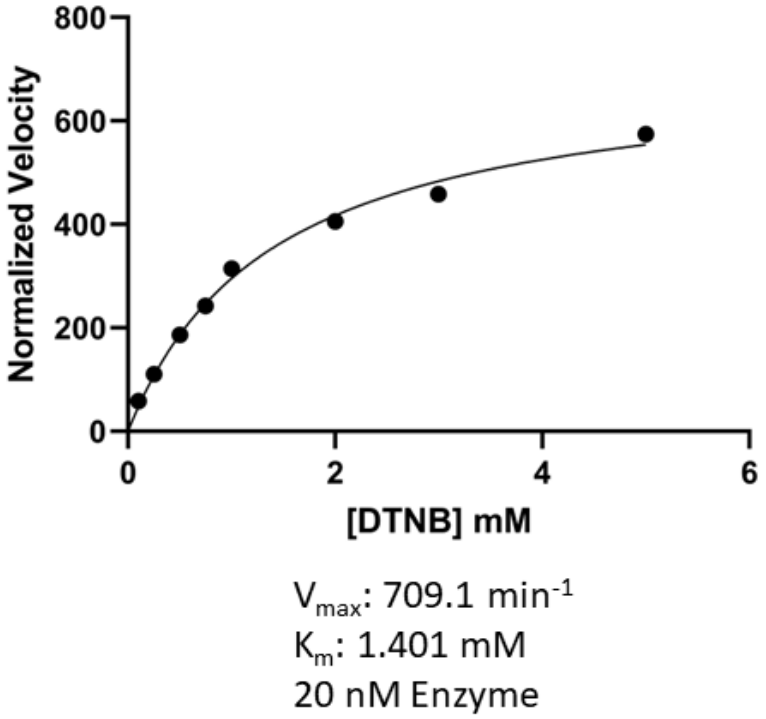
Supp. Figure 3: Michaelis-Menten curves of Mutant 3 (PfTrxR – CUGGK2THG) Activity Assays. (a) Thioredoxin assay with 13 nM enzyme. Normalized velocity is moles NADPH/min/mole PfTrxR. (b) H_2O_2 assay with 30 nM enzyme. Normalized velocity is moles NADPH/min/mole PfTrxR. (b) Ellman's reagent (DTNB) assay with 8nM enzyme. Normalized velocity is moles TNB/min/mole PfTrxR.



Supp. Figure 4: Michaelis-Menten curves of Mutant 4 (PfTR – CUGGKSG) Activity Assays. (a) Thioredoxin assay with 10 nM enzyme. Normalized velocity is moles NADPH/min/mole PfTrxR. (b) H_2O_2 assay with 30 nM enzyme. Normalized velocity is moles NADPH/min/mole PfTrxR. (b) Ellman's reagent (DTNB) assay with 2 nM enzyme. Normalized velocity is moles TNB/min/mole PfTrxR.



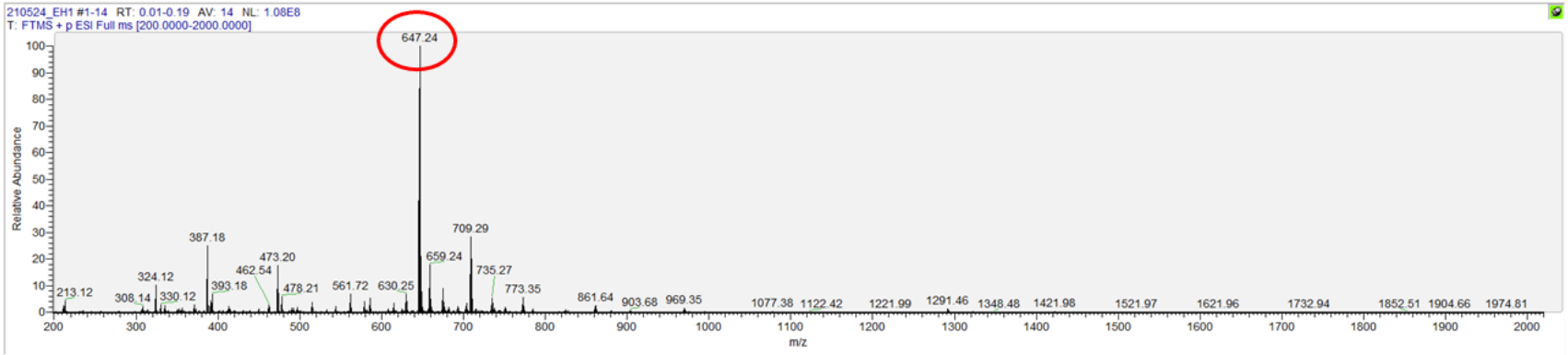
Supp. Figure 5: Michaelis Menten curves of Enzyme 5 (PfTrxR – CGGGKCG, Wild-Type) Thioredoxin Activity Assay. Enzyme concentration is 45 nM. Normalized velocity is moles NADPH/min/mole PfTrxR.



Supp. Figure 6: Michaelis Menten Curves of Enzyme 6 (PfTrxR - Δ7, Truncated) Ellman's Reagent (DTNB) Activity Assay. Enzyme concentration is 20 nM. Normalized velocity is moles TNB/min/mole PfTrxR.

CGGGK2THG

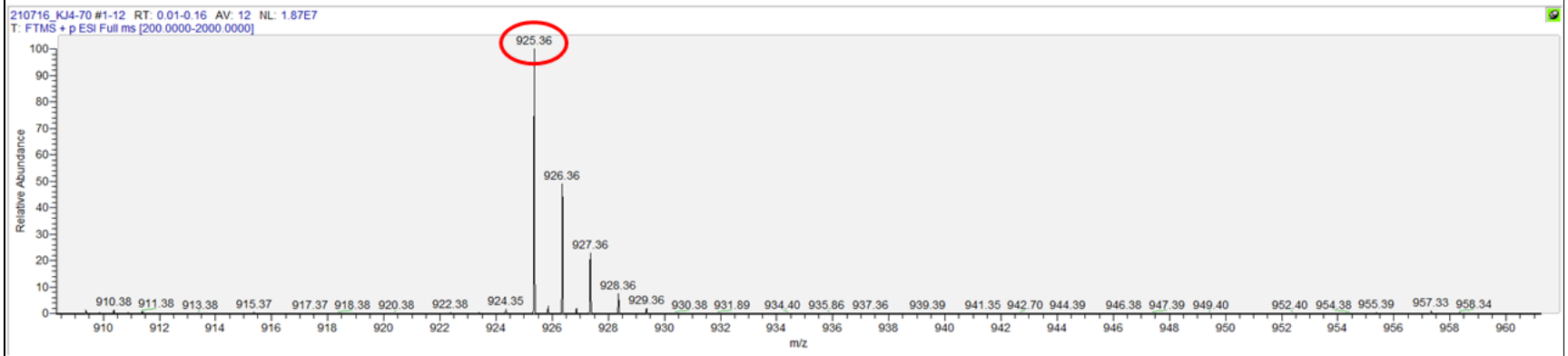
MW: 646 g/mol



Supp. Figure 7: Direct Inject Mass Spectroscopy of Mutant 1 Peptide (CGGGK2THG). MW 646 g/mol, peak confirming presence of correct peptide at 647.24 (circled in red).

CG2THKPG2THK

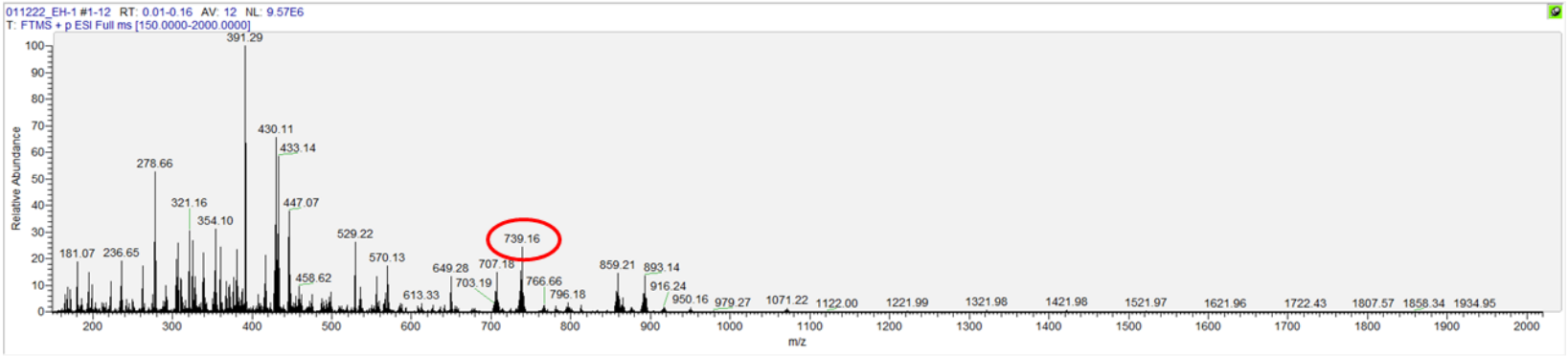
MW: 926.5 g/mol



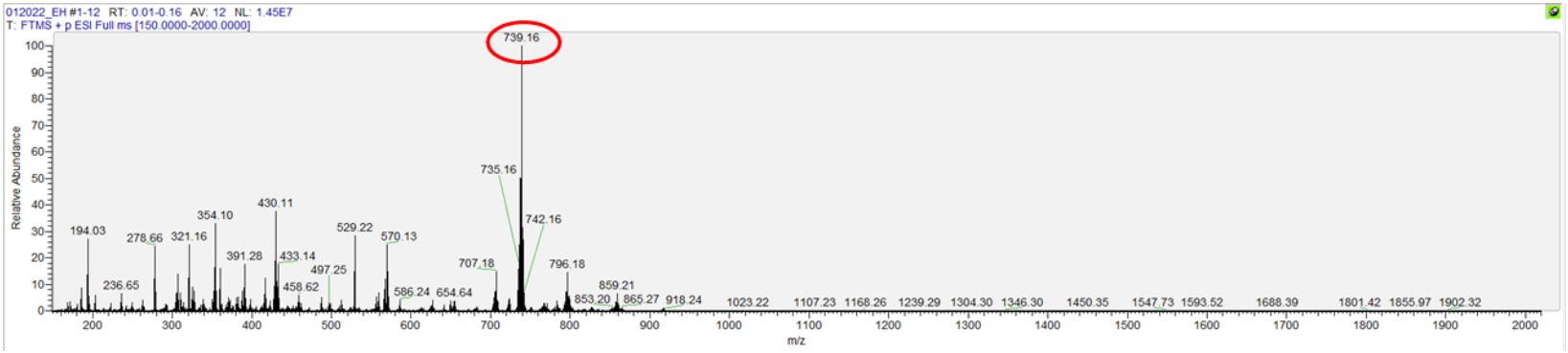
Supp. Figure 8: **Direct Inject Mass Spectroscopy of Mutant 3 Peptide (CG2THKPG2THK).** MW: 926.5 g/mol, peak confirming presence of correct peptide at 925.36 m/z (circled in red).

CUGGK2THG

MW: 739 g/mol



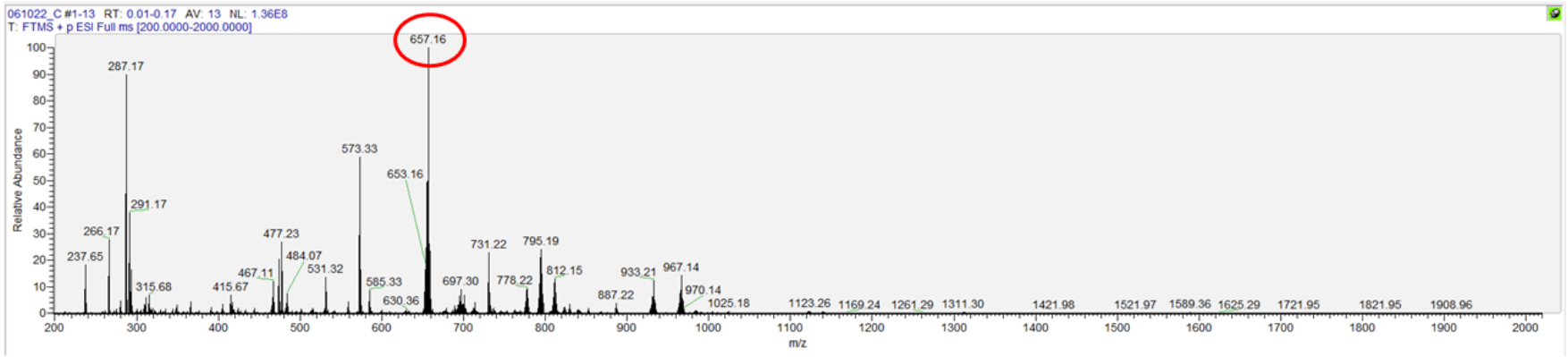
After Deprotection of Selenocysteine:



Supp. Figure 9: Direct Inject Mass Spectroscopy of Mutant 3 Peptide (CUGGK2THG). MW 739 g/mol, peak confirming presence of correct peptide at m/z 739.16 (circled in red). Bottom shows mass spectroscopy of peptide sample after deprotection of Mob, see increase in relative abundance of m/z 739.16 (circled in red).

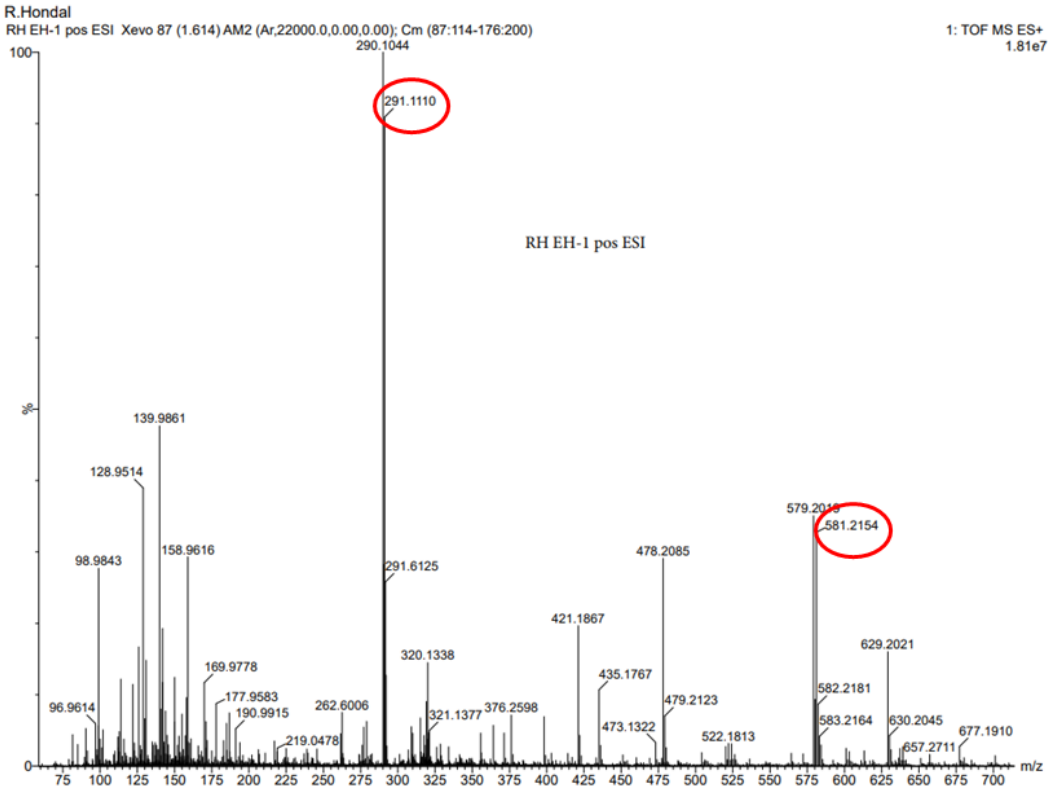
CUGGKSG

MW: 657 g/mol



Supp. Figure 10: Direct Inject Mass Spectroscopy of Mutant 4 Peptide (CUGGKSG). MW 657 g/mol, peak confirming presence of correct peptide at m/z 657.16 (circled in red).

CGGGKCG
MW: 581 g/mol



Supp. Figure 11: Direct Inject Mass Spectroscopy of Enzyme 5 Peptide (CGGGKCG, Wild-type). MW 581 g/mol, peak confirming presence of correct peptide at m/z 581.21 (circled in red). Doubly charged ion at m/z 291.11 (circled in red).