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A “seleno-effect” differentiates the roles of redox active cysteine residues in *Plasmodium falciparum* thioredoxin reductase[†]

John P. O’Keefe^{§,†}, Christopher M. Dustin[‡], Drew Barber^{¶,‡}, Gregg W. Snider[‡], and Robert J. Hondal^{*,‡}

[‡]Department of Biochemistry, 89 Beaumont Ave, Given Building Room B413, Burlington, VT 05405

Abstract

Here, we introduce the concept of the “seleno effect” in the study of oxidoreductases that catalyze thiol/disulfide exchange reactions. In these reactions, selenium can replace sulfur as a nucleophile, electrophile, or leaving group, and the resulting change in rate (the seleno effect) is defined as k_S/k_{Se} . In solution selenium accelerates the rate of thiol/disulfide exchange regardless of its chemical role (e.g. nucleophile or electrophile). Here we show that this is not the case for enzyme catalyzed reactions and that the magnitude of the seleno effect can differentiate the role of each sulfur atom of a disulfide bond between that of an electrophile or leaving group. We used selenium for sulfur substitution to study the thiol/disulfide exchange step that occurs between the N-terminal redox center and the C-terminal disulfide-containing β -hairpin motif of *Plasmodium falciparum* thioredoxin reductase (PfTrxR), which has the sequence Gly-Cys⁵³⁵-Gly-Gly-Gly-Lys-Cys⁵⁴⁰-Gly. We assayed a truncated PfTrxR enzyme missing this C-terminal tail for disulfide-reductase activity using synthetic peptide substrates in which either Cys⁵³⁵ or Cys⁵⁴⁰ was replaced with selenocysteine (Sec). The results show that substitution of Cys⁵³⁵ with Sec resulted in a nearly 9-fold decrease in the rate of reduction, while substitution of Cys⁵⁴⁰ resulted in a 1.5-fold increase in the rate of reduction. We also produced full-length, semisynthetic enzymes in which Sec replaced either of these two Cys residues and observed similar results using *E. coli* thioredoxin as the substrate. In this assay the observed seleno effect (k_S/k_{Se}) for the C535U mutant was 7.4, and 0.2 for the C540U mutant.

Graphical Abstract

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^{*}To whom correspondence should be addressed: Department of Biochemistry, University of Vermont, College of Medicine. 89 Beaumont Ave, Given Building Room B413, Burlington, VT 05405. Tel: 802-656-8282. FAX: 802-656-8220. Robert.Hondal@uvm.edu.

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SUPPORTING INFORMATION AVAILABLE

There are six figures in the Supporting Information. Figures S1-S5 are mass spectrograms of the synthetic peptides used in this study. Figure S6 is a plot of velocity versus substrate concentration from 0 to 1 mM substrate. This material is available free of charge *via* the Internet at <http://pubs.acs.org>

INTRODUCTION

A useful tool to study the mechanism of phosphohydrolases and phosphotransferases is through the use of thio substitution for one of the substrate phosphoryl oxygen atoms (1–5). This substitution results in a change in the rate of reaction at phosphorous (k_O/k_S), and this change in rate has been termed the “thio effect” (1).

In this study we define an analogous “seleno effect” (k_S/k_{Se}), albeit for thiol/disulfide exchange reactions instead of reactions involving attack of phosphodiester. Selenium is the atom directly underneath sulfur on the periodic chart and is chemically similar, although there are significant distinctions (6). Substitution of selenium for sulfur in a thiol/disulfide exchange reaction in solution accelerates the rate by one of three ways: (i) a selenolate is a better attacking nucleophile than a thiolate, (ii) a selenolate is a better leaving group than a thiolate, and (iii) selenium in a selenosulfide bond is more electrophilic than sulfur (7–11).

While substitution of selenium for sulfur in a thiol/disulfide exchange reaction is expected to accelerate the rate of reaction *in solution* (7, 11), we wanted to test whether this would be the case in an *enzyme catalyzed reaction* where the asymmetry of an enzyme active site might modify the effects of the substitution on the rate of the reaction. To test the “seleno effect” in an enzyme catalyzed reaction, we chose the thiol/disulfide exchange step that occurs between the N- and C-terminal redox centers of the high molecular weight (M_r)¹ thioredoxin reductase (TrxR) from *Plasmodium falciparum* (PfTrxR).

¹ABBREVIATIONS AND TEXTUAL FOOTNOTES

A₃₄₀, absorbance at 340 nm; βME, beta-mercaptoethanol; CBD, chitin-binding domain; C, one-letter abbreviation for cysteine; Cys, cysteine; Cys_{IC}, interchange cysteine; DCM, dichloromethane; DEAE, diethylaminoethyl; DMF, *N,N*-dimethyl formamide; *D. melanogaster*, *Drosophila melanogaster*; DmTrxR, *Drosophila melanogaster* thioredoxin reductase; DTNB/Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; FADH₂, flavin adenine dinucleotide reduced; Fmoc, 9-fluorenylmethoxycarbonyloxy; Fmoc-AA-OH, *N*-Fmoc-amino acids; G, one-letter abbreviation for glycine; Gly, glycine; HPLC, high pressure liquid chromatography; His, histidine; hCys, homocysteine; 1-hydroxy-7-azabenzotriazole, HOAt; K, one-letter abbreviation for lysine; lysine, Lys; mTrxR, mouse mitochondrial thioredoxin reductase; M_r , molecular ratio; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NADPH, β-nicotinamide adenine dinucleotide phosphate-reduced; NMA, *N*-methylmercaptoacetamide; NMM, *N*-methyl morpholine; OD600, optical density at 600 nm; PfTrxR, *Plasmodium falciparum* thioredoxin reductase; S, one-letter abbreviation for serine; S, sulfur; Se, selenium; Sec, selenocysteine; Ser, serine; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Tris, tris-(hydroxymethyl)aminomethane; Trx, thioredoxin; TrxR, thioredoxin reductase; U, one-letter abbreviation for selenocysteine; UV-Vis, ultraviolet-visible; WT, wild type; Xan, xanthene.

PfTrxR, like other high M_r thioredoxin reductases is a homodimer that contains a bound flavin that accepts reducing equivalents from NADPH. The reduced flavin transfers two electrons to a N-terminal CVNVGC disulfide (N-terminal redox center), resulting in a charge-transfer complex and a reduced thiolate (Cys_{IC}) that is poised for nucleophilic attack onto a C-terminal β -hairpin motif of sequence GCGGGKCG as shown in Figure 1 (12).

The thiol/disulfide exchange step between N- and C-terminal redox centers was isolated by synthesizing an oxidized, disulfide-containing peptide with the same amino acid sequence as found in the native, holoenzyme, which was then used as a “substrate” for the truncated PfTrxR missing this sequence (Figure 1). This approach allows us to focus on this individual step in the enzyme mechanism, and replacement of either Cys⁵³⁵ or Cys⁵⁴⁰ of the β -hairpin motif with selenocysteine (Sec) allows us to measure the effect of selenium for sulfur substitution on rate.

We also synthesized full-length, semisynthetic selenocysteine-containing enzymes in which Sec replaced each cysteine (Cys) residue of the C-terminal redox motif using protein semisynthesis (13). We assayed these Sec-containing semisynthetic enzymes for thioredoxin reductase activity using *E. coli* thioredoxin (Trx), and an alternative substrate, selenocystine. Selenocystine is a diselenide-containing molecule that requires an intact C-terminal redox center in order for it to be reduced by the enzyme (14, 15). Both of these assays revealed seleno effects for both Cys⁵³⁵ and Cys⁵⁴⁰ that were very similar to our assays using the truncated enzyme and peptide substrates.

PfTrxR is a Cys-ortholog of the Sec-containing mammalian TrxR that contains a much different C-terminal redox center, Gly-Cys-Gly-Gly-Gly-Lys-Cys-Gly (GCGGGKCG), in comparison to the mammalian enzyme, which contains a Gly-Cys-Sec-Gly (GCUG) motif. PfTrxR and other Cys-ortholog TrxRs are capable of nearly as efficient reduction of their cognate Trx substrates (16). Replacement of Sec with Cys in the mammalian enzyme results in a large loss in activity. This loss in activity is due at least in part to the C-terminal disulfide of the mutant (GCCG) being much less reactive towards thiol/disulfide exchange compared to the C-terminal selenosulfide (GCUG) that is found in the wild type (WT) enzyme as determined previously by similar experiments (17). The C-terminal disulfide of Cys-ortholog TrxRs must be made more reactive in some way so that these Cys-orthologs have similar activity compared to the Sec-containing enzymes.

We have previously put forth a model to explain how the C-terminal disulfide of a related TrxR from *D. melanogaster* (DmTrxR) can be made reactive towards thiol/disulfide exchange. We termed this model as “electrophilic activation”, because we provided evidence that the C-terminal disulfide becomes more electrophilic in the enzyme than it would be in solution, and hence more reactive towards thiol/disulfide exchange (18). The seleno effects we have found in PfTrxR support a similar model of activation of the C-terminal disulfide, even though the PfTrxR disulfide sequence, GCGGGKCG, is much different from the DmTrxR C-terminal disulfide sequence, Ser-Cys-Cys-Ser (SCCS).

Our model is supported by further experiments in which each of the Cys residues of the β -hairpin of PfTrxR were replaced with homocysteine (hCys). Replacement of either Cys

residue with hCys of a peptide substrate resulted in significantly reduced activity compared to the WT peptide, and this result is also consistent with our electrophilic activation model.

Our overall results have helped to differentiate the catalytic role of each Cys residue of the C-terminal redox center of PfTrxR, and we hope that our development of the seleno effect will be useful in the study of oxidoreductases that catalyze thiol/disulfide exchange reactions.

MATERIALS AND METHODS

Materials

NADPH was purchased from AppliChem (Darmstadt, Germany). DEAE resin was obtained from Sigma-Aldrich (St. Louis, MO). 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). Solvents for peptide synthesis were purchased from EMD Biosciences (San Diego, CA). All peptides were synthesized with a Burrell Model 75 wrist-action shaker (Pittsburgh, PA). *N*-Fmoc-amino acids were purchased from Synpep Corp (Dublin, CA), except for Fmoc-homocysteine, which was from Bachem (King of Prussia, PA) and Fmoc-Sec-Xanthene, which was obtained as a gift from Dr. Stephenson Flemer (19). Microcon Ultracel YM-50 ultrafiltration devices by Millipore (Billerica, MA) were used for concentrating enzyme samples. 2-chlorotriylchloride resin was from Novabiochem (San Diego, CA). CLEAR-OX resin was from Peptides International (Louisville, KY). Mass spectral analysis was performed on an Applied Biosystems QTrap 4000 hybrid triple-quadrupole/linear ion trap liquid chromatograph-mass spectrometer (SciEx, Framingham, MA). Enzyme kinetic assays were performed on a Cary50 UV-Vis spectrophotometer (Walnut Creek, CA) and all enzymatic assays were conducted at room temperature unless otherwise noted. All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI), Fisher Scientific (Fair lawn, NJ), or Acros Organics (Morris Plains, New Jersey).

Peptide synthesis

Peptides were synthesized on a 0.3 mmol scale using 2-chlorotriylchloride resin (1.33 mmol/g loading) with 3-fold excess resin used relative to the peptide synthesis scale. 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 (equivalents relative to the peptide synthesis scale in mmoles). Fmoc-AA-OH was dissolved in 8 mL of a 2.2% *N*-methylmorpholine (NMM)/97.8% DCM (0.2 M NMM) solution and shaken for 1 h. The resin was then washed 4x using DCM. Following this washing step, remaining unreacted sites on the resin were capped by reaction with 10 mL of 8:1:1-DCM:methanol:NMM solution followed by shaking for 15 min. After completion of this capping step, the resin was washed 4x with DCM and then 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 test (20). Elongation of the peptides used standard Fmoc SPPS chemistry with 2 eq. HATU (*O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate) and 2 eq. Fmoc-AA-OH in 8 mL of a 4.40%

NMM/95.6% DMF (0.5 M NMM) solution, shaken for one h (21). Upon completion of the elongation steps, a final deprotection of Fmoc was performed, after which the resin was washed with DCM (4x). Cleavage of the peptides from the solid support was done with a cocktail consisting of 96:2:2 -trifluoroacetic acid (TFA):triisopropylsilane (TIS):H₂O for 2 h. The cleavage solution was collected into a conical tube using nitrogen gas and the resin was washed with DCM (4x). The cleavage solution was then concentrated by evaporation using a stream of nitrogen over the surface of the solution. The peptides were first precipitated by dripping the cleavage solution into cold, anhydrous diethyl ether and then collected by centrifugation. The pellet was allowed to air dry after which it was dissolved again into 1 mL of the 96:2:2 TFA:TIS:H₂O cleavage cocktail and incubated for a further 1 h at room temperature to ensure complete removal of protecting groups. The peptide was then precipitated by addition of the cocktail into cold, anhydrous diethyl ether, followed by centrifugation to pellet the peptide. The pellet was then washed with anhydrous diethyl ether and pelleted by centrifugation (2x). The pelleted peptide was then dissolved in a minimal amount of a water/acetonitrile mixture and lyophilized.

Syntheses of selenocysteine-containing peptides

The Fmoc-Sec-xanthene derivative was used for the installation of the Sec residue into peptides H-GCGGGKUG-OH (peptide **2**) and H-GUGGGKCG-OH (peptide **3**) synthesized in this study (19). The resin and procedure for these peptides was the same as described previously, with one exception. When coupling Fmoc-Sec-Xan and all of the amino acids following it in the synthesis the conditions were altered to avoid racemization and other unwanted side reactions (21). The conditions for these couplings were as follows: 2 eq Fmoc-AA-OH and 4 eq 1-hydroxy-7-azabenzotriazole (HOAt) coupling reagent were dissolved in 5 mL of 1.88% diisopropylcarbodiimide (DIC)/98.1% DMF (94 μ L/4.906 mL) and shaken. The coupling of Fmoc-Sec(Xan) was for 3 h, but subsequent couplings of other amino acids were performed for 1 h. The cleavage and swelling of the resin used the same procedure as for the cysteine amino acids. The oxidation of the Sec peptides occurs during cleavage from the resin. The xanthene protecting group is 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 resulting in a selenosulfide bond. This was confirmed by MS analysis of the peptide (Figures S1 and S2 of the Supporting Information). No further purification of the peptide was required.

Oxidation of wild type peptide

During the synthesis of the WT peptide (H-GCGGGKCG-OH, peptide **1**) Fmoc-Cys-*S*-Tnp was used as the cysteine building block in both locations along the peptide. After the final coupling the final *N*-Fmoc group was left intact and the peptide was oxidized to form a disulfide bond directly while still attached to the solid support. To do this 0.1 M NMM in 5% dithiothreitol (DTT)/95% DMF was first added to the resin and shaken for 5 min (3x) to deprotect the *S*-Tnp group (22). The resin was then washed with DMF (4x) followed by washing with DCM (4x). The success of the deprotection was tested using a solution of Ellman's reagent in DCM on a sample of the resin beads. Successful deprotection of the *S*-Tnp group results in a free Cys-SH that will react with Ellman's reagent and results in a visible yellow color (23). After completion of *S*-Tnp deprotection and the Ellman's test, the

resin was washed with DMF (4x) and 2 eq of *N*-chlorosuccinimide dissolved in 5 mL of DMF was added to the resin and shaken for 15 min to form the disulfide bond (22). After washing with DMF (4x) and DCM (4x) the resin was subjected to Ellman's test to confirm the oxidation. In this case, the solution should remain clear because no free Cys-SH groups are present. The final Fmoc group was then removed with piperidine and the peptide was cleaved from the resin as described above. The oxidation of the peptide to the disulfide form was confirmed by MS analysis (Figure S3 of the Supporting Information).

Oxidation of the homocysteine-containing peptides

Each Cys residue of the WT peptide was replaced with hCys to produce peptides H-GCGGGK**h**CG-OH (peptide **4**) and H-G**h**CGGGKCG-OH (peptide **5**). Unlike the other peptides in this study, the two homocysteine-containing peptides were oxidized after cleavage from the resin. CLEAR-OXTM resin was used to induce disulfide formation of the disulfide bond in these peptides (24). This resin has 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB/Ellman's reagent) bound to CLEAR resin. The bound DTNB catalyzes disulfide bond formation in peptide/protein solutions. The CLEAR-OXTM resin was loaded at a 0.21 meq/g of resin:peptide ratio, 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. Each homocysteine-containing peptide was then dissolved in 1:1 acetonitrile: 0.1 M degassed ammonium bicarbonate buffer (1 mL of solution per 6 mg of peptide). The solution was added to the resin and agitated for 2 h. Following agitation, the solution was blown out of the vessel with nitrogen 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. To remove these impurities, the lyophilized peptide was dissolved in 10 mM ammonium biocarbonate, pH 7.5, and then added to a slurry of DEAE-Sepharose resin equilibrated in the same buffer. The impurities are negatively charged at this pH, while the peptide is positively charged and unable to bind the resin. After agitating the sample with the resin for 30 min, the resin slurry was filtered to remove the resin. The eluent containing the peptide was then lyophilized and subjected to MS analysis to verify the identity and purity of the peptide (Figures S4 and S5 of the Supporting Information).

Production of semi-synthetic mutants of PfTrxR

Two semisynthetic Sec-containing enzymes were produced in this study. They are abbreviated as C535U and C540U, to indicate the position of each Cys residue that was replaced with Sec. The production of these semisynthetic enzymes was achieved through intein-mediated peptide ligation (13, 25). First, a truncated PfTrxR construct missing seven residues from the C-terminus, PfTrxR₇, was formed through PCR amplification of the full-length wild type PfTrxR DNA template. An upstream primer which 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 MastercyclerTM using Vent DNA polymeraseTM. 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 PfTrxR 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 µg/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 equilibrated in chitin buffer A [50 mM MOPS and 150 mM NaCl (pH 7.0)] followed by cell lysis with 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 PfTrxR 7 allowed it to bind to the resin with high specificity. The loaded PfTrxR 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 added to the resin containing the bound protein to convert it to slurry.

The semisynthetic PfTrxR enzymes were produced via ligation of desired synthetic peptides to the truncated PfTrxR 7 containing a C-terminal thioester reactive group. The peptide bond between the final Cys residue of the enzyme and the intein rearranges to form a thioester, which can be cleaved to remove the intein and form a different thioester. Proteins containing a C-terminal thioester can be ligated to peptides that have an N-terminal Cys (or Sec) residue to form an amide bond (25). Simultaneous cleavage of the fusion protein from the intein 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 [1 M NH₄SO₃, 50 mM Tris pH 7.5] via ultrafiltration (Amicon Ultracel 30 kDa cut off filters). It was then loaded by gravity onto a pre-equilibrated phenyl sepharose column (20 mL). After the sample was washed with buffer C, the semisynthetic PfTrxR enzyme was eluted from the phenyl sepharose column with buffer lacking NH₄SO₃ [50 mM Tris pH 7.5], to disrupt binding to the column. The fractions containing PfTrxR were confirmed by spectrophotometry and SDS-PAGE, pooled, and then buffer exchanged through ultrafiltration with storage buffer [50 mM potassium phosphate, 500 mM NaCl, and 1 mM EDTA (pH 8.0)]. The concentration of each PfTrxR 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).

Production of the PfTrxR 7 truncated enzyme

PfTrxR 7 was used to assay synthetic peptide disulfide substrates described above. This truncated PfTrxR was made using the procedure previously described for the production of the PfTrxR 7-intein-chitin binding domain construct. After allowing this protein to bind to the chitin column the enzyme was cleaved from the intein using 120 mM NMA in chitin buffer B without peptide present. Following cleavage from the column, PfTrxR 7 fractions were pooled and concentrated using ultrafiltration (Amicon Ultracel 30 kDa filters). The sample was then loaded onto a Sephacryl S-200 column pre-equilibrated with 50 mM potassium phosphate, 500 mM sodium chloride, and 1 mM EDTA (pH 8.0). Fractions of the elution that contained PfTrxR 7 were then pooled and concentrated using the methods above.

Peptide substrate assays

Enzyme assays were performed using synthesized oxidized peptides as substrates for the truncated PfTR 7 enzyme. The conditions for each assay were 500 μ L total volume, 1 mM EDTA, 100 mM potassium phosphate, pH 7.0 buffer, 150 μ M NADPH, 15 nM PfTrxR 7, and varying concentrations of peptides. The consumption of NADPH by PfTrxR 7 was measured at 340 nm using a spectrophotometer (Cary-50).

Enzymatic assays of semisynthetic PfTrxR

Semisynthetic enzyme assays were performed using the ligated enzymes PfTrxR-GUGGGKCG and PfTrxR-GCGGGKUG with selenocystine or *E. coli* thioredoxin (Trx) as substrates. The conditions of the assays were 1 mL total volume, 200 μ M NADPH, 500 mM potassium phosphate, pH 7.0 buffer, 10 mM EDTA, and 100 nM semisynthetic enzyme with either Trx or selenocystine as substrates with varying concentrations. The consumption of NADPH by the enzymes was measured at 340 nm using a spectrophotometer (Cary-50).

Measuring Trx-reductase activity of Sec-containing PfTrxRs after incubation with NADPH and H₂O₂

In order to directly compare the ability of the WT PfTRxR and the Sec-containing mutant PfTrxRs to resist peroxide-induced inactivation we developed a two part assay in which we first exposed the reduced enzymes to increasing concentrations of H₂O₂ (0–50 mM) and then assessed the remaining Trx reductase activity relative to a control reaction where H₂O₂ was omitted. For this assay we used 45 nM enzyme for both WT PfTrxR and PfTrxR-GCGGGKUG, but 100 nM enzyme for PfTrxR-GUGGGKCG due the significantly overall lower activity of this mutant enzyme. These enzymes were first reduced with NADPH (200 μ M) for 5 min, followed by the addition of increasing amounts of H₂O₂ (0–50 mM) to the 0.7 mL reaction mixture also containing 1 mM EDTA in 100 mM potassium phosphate buffer, pH 7.0. The enzymes were incubated with oxidant for 25 min upon which catalase (14 Units) was added to the reaction to quench excess peroxide in an additional 12 min reaction. An additional bolus of 200 μ M NADPH followed by 90 μ M *E. coli* Trx were next added to the reaction and the Trx-reductase activity was measured by monitoring the consumption of NADPH as a decrease in A₃₄₀. Background non-enzymatic or Trx-independent consumption of NADPH was subtracted from the experimental activities by

carrying out control experiments in which either enzyme or Trx was omitted from the reaction. The change in absorbance was converted to specific activity (mol of NADPH • min⁻¹ • mol of PfTrxR⁻¹) and the ability to resist oxidant-induced inactivation was quantified as a percentage of activity remaining relative to the oxidant-untreated control. Conditions reported here are nearly identical to those reported in (26).

RESULTS

Activity of PfTrxR 7 with synthetic peptide-disulfide substrates

The synthesized disulfide-or selenosulfide-containing peptides were used as substrates for PfTrxR 7 in an assay that monitors the consumption of NADPH at 340 nm and directly reflects the rate of thiol/disulfide exchange between the N-terminal redox center of PfTrxR 7 and the peptide substrate (Figure 1).

Plots of activity (mol NADPH consumed per min per mol of PfTrxR 7) versus peptide substrate concentration for the Sec-containing peptides is shown in Figure 2A, and the same activity plot using the hCys-containing peptides is shown in Figure 2B. A seleno effect is clearly visible in Figure 2A since peptide 2 is reduced faster than peptide 1 at all peptide concentrations, while peptide 3 is reduced much slower than peptide 1 at all peptide concentrations (see Table 1 for assignment of peptide numbers). The overall data from these plots is summarized in Table 1.

Ideally the seleno effect could be determined by calculating the 2nd order rate constant (k_{cat}/K_M – catalytic efficiency) for each peptide substrate. The ratio of k_{cat}/K_M for peptide 1 to the k_{cat}/K_M of either of the Sec-containing peptides would approximate k_S/k_{Se} . Since peptide 2 did not display saturation kinetics, we could not calculate this ratio for both Sec-containing peptides. Instead we quantified the seleno effect by two methods, which yielded qualitatively similar values. First, the ratio of activities of peptide 1 to either of the Sec-containing peptides at high peptide concentration (5 mM) gives values of 0.58 for peptide 2, and 4.6 for peptide 3. Second, we calculated V/K (V_{max}/K_M) for each peptide substrate (Table 1) by calculating the slope of the linear part of the plot of velocity versus peptide concentration shown in Figure 2A by only considering the velocity between 0 and 1 mM peptide. This plot is shown in Figure S5 of the Supporting Information. The ratio of V/K values is equivalent to the seleno effect and quantifies the amount of rate acceleration or rate deceleration when Sec replaces Cys.

We also replaced each Cys residue of the WT-peptide with hCys, the side chain of which is one methylene unit longer than Cys. This replacement does not alter the peptide backbone, but makes a subtle change to the size of the disulfide ring connecting the two β -strands from 20 atoms to 21 atoms. The increase in disulfide ring size slows the rate of thiol/disulfide exchange by 5.4-fold for peptide 4 (hCys replaces Cys⁵⁴⁰) and 3.5-fold for peptide 5 (hCys replaces Cys⁵³⁵) as determined by calculating the ratio of activities at 5 mM peptide concentration. Thus, substitution of hCys for either Cys residue slows the rate of thiol/disulfide exchange by a similar magnitude.

Semisynthetic enzyme assays

Two semisynthetic mutants of PfTrxR were created in which Sec replaces Cys. The PfTrxR-GUGGGKCG (Cys⁵³⁵→Sec mutation) enzyme is abbreviated as C535U and the PfTrxR-GCGGGKUG (Cys⁵⁴⁰→Sec mutation) enzyme is abbreviated as C540U. Both of these mutant enzymes and WT PfTrxR were assayed for Trx-reductase and selenocystine-reductase activity. Plots of these activity assays as shown in Figure 3A and Figure 3B, respectively, and the data are summarized in Tables 3 and 4.

A seleno effect is clearly demonstrated by the kinetic data for both Sec-containing mutant enzymes, but just as in the assays with peptide substrates, the magnitude is quite different. The C540U mutant shows an inverse seleno effect of 0.2 ($k_S/k_{Se} < 1$) with *E. coli* Trx as the substrate and an inverse seleno effect of 0.16 with selenocystine as the substrate. In contrast, the C535U mutant shows normal seleno effects for *E. coli* Trx and selenocystine, of 7.4 and 3.7, respectively ($k_S/k_{Se} > 1$). As was the case with assays with the peptide substrates, replacement of Cys⁵⁴⁰ with Sec accelerates the rate of the reaction for both substrates, while replacement of Cys⁵³⁵ with Sec slows the reaction rate for both substrates.

We note that the production of all semisynthetic proteins result in incomplete ligation. Thus, the semisynthetic, Sec-containing enzymes in this study are a mixture of truncated and full-length enzyme and we have not quantified the extent of ligation. However, we have used two different approaches to measure the seleno effect and the results from both methods reinforce each other.

In the first approach, we used truncated PfTrxR as a catalyst and a synthetic peptide as substrate. Each reagent was close to 100% purity. As illustrated in Figure 2A, a clearly visible seleno effect shows that the rate acceleration or deceleration is due to the position of the selenium atom in the selenosulfide bond of the peptide substrate. In the second approach we measured very similar seleno effects using either *E. coli* Trx or selenocystine as substrates when Sec occupied the identical position in the semisynthetic enzyme as it did in the peptide substrate. As both results are in agreement, this give us confidence that the observed changes in kinetic parameters in the semisynthetic enzymes relative to the WT enzyme are due to the position of Sec in the C-terminal redox center, and not due to largely incomplete ligation.

In examining the individual kinetic parameters for each enzyme and substrate, it can be seen that the WT enzyme shows a high K_M for *E. coli* Trx, a non-cognate substrate. When the cognate Trx from *P. falciparum* is used, the K_M is ~30-fold lower (27). Replacement of either Cys residue of the C-terminal redox center with Sec results in much lower K_M values. The K_M for the C540U mutant is 18-fold lower compared to the WT enzyme, while the K_M for the C535U mutant is 50-fold lower. With selenocystine as the substrate, the change in K_M is smaller, 9- and 11-fold, respectively.

The change in both k_{cat} and K_M for the C540U mutant compared to the WT enzyme merits special comment. A visual inspection of Figure 3A shows that the decrease in K_M for *E. coli* Trx is much greater (18-fold) relative to the small decrease (3.7-fold) in k_{cat} . As a result, the C540U mutant has higher specific activity at lower substrate concentrations because the

mutant reaches a $V_{\max}$ near 150 μM of Trx, while the WT does not reach $V_{\max}$ until the concentration of Trx is $\sim 6000 \mu\text{M}$. Obviously the C540U mutant is saturated at much lower concentration of substrate. Consequently, the k_{cat}/K_M of the C540U mutant is 5-fold higher (inverse seleno effect) compared to the WT enzyme.

The rate constant k_{cat} decreases for both Sec-mutant enzymes with both types of substrates examined here, but the decrease in k_{cat} is much lower for the C535U mutant compared to the C540U mutant. For the C540U mutant, the decrease is 3.8 and 1.5-fold for Trx and selenocystine, respectively. In contrast the decreases in k_{cat} for the C535U mutant for the same substrates are 355 and 41-fold, respectively.

An additional seleno effect is observed with respect to resistance to oxidative inactivation

Since the substitution of each Cys residue of the C-terminal redox center with Sec resulted in significant changes in catalytic parameters, we also wanted to test whether replacement of the Cys residues of the C-terminal redox center with Sec would enable the mutant enzymes to maintain activity after exposure to oxidants, as has been observed for other enzymes in which Sec has replaced Cys (26, 28, 29). We first reduced the WT and Sec-containing enzymes with NADPH and then incubated them with increasing concentrations of H_2O_2 for 25 min. After removal of any remaining H_2O_2 by catalase, we assessed each enzyme's ability to reduce *E. coli* Trx (90 μM) compared to when no H_2O_2 was present. The results of this assay for the WT and the Sec-containing mutant enzymes are shown in Figure 4.

As can be seen from the plot of activity remaining versus H_2O_2 concentration, the ability of both Sec-containing mutant enzymes to retain activity after H_2O_2 treatment is increased compared to the WT enzyme. While the C535U mutant retains more activity compared to the WT enzyme at low concentrations of H_2O_2 , the effect is greatly diminished at higher concentrations. This is in contrast to the C540U mutant, which retains much of its activity even at 50 mM H_2O_2 . These data show that there is an additional "seleno effect" with respect to the ability of the mutant enzymes to retain activity after exposure to oxidant, since Sec for Cys substitution has a more pronounced effect on resistance to oxidative inactivation at the C-terminal position of the redox center compared to the N-terminal position.

DISCUSSION

Interpretation of the "seleno effect" and comparison to the well described "thio effect"

As introduced earlier, substitution of sulfur for an oxygen atom in a phosphodiester leads to a change in reaction rate in substitution and hydrolysis reactions referred to as a thio effect ($k_{\text{O}}/k_{\text{S}}$). Thio effects are of two types: A regular thio effect ($k_{\text{O}}/k_{\text{S}} > 1$) occurs when sulfur substitutes for a non-bridging oxygen and is caused by overall transition state destabilization due in part to less efficient solvation of sulfur, while an "inverse" thio effect ($k_{\text{O}}/k_{\text{S}} < 1$) is due to the better leaving group ability of an alkylthiolate relative to an alkoxide.

Here, we have introduced the concept of an analogous "seleno effect" ($k_{\text{S}}/k_{\text{Se}}$) in oxidoreductases that catalyze thiol/disulfide exchange reactions in which selenium replaces sulfur. Similar to a thio effect, a seleno effect can be "normal" ($k_{\text{S}}/k_{\text{Se}} > 1$) or "inverse" ($k_{\text{S}}/k_{\text{Se}} < 1$) as demonstrated by the data presented here.

Previous work by Koppenol and coworkers made a detailed study of thiol/disulfide exchange reactions using a cysteamine/cystamine model system (7). Replacement of cystamine with selenocystamine in their reaction resulted in a selenium for sulfur substitution and is of great interest here in comparison to our system using an enzyme and peptide disulfide/selenosulfide substrates. The seleno effect (k_S/k_{Se}) calculated with their data is reported in Table 4.

Selenium for sulfur substitution in thiol/disulfide exchange reactions accelerates the rate of reaction whether it is acting a nucleophile, electrophile, or leaving group as can be seen by the magnitude of k_S/k_{Se} given in Table 4 for each of these scenarios. As noted by Koppenol, the greatest rate acceleration is observed when selenium acts as an electrophile and this is responsible for 10^2 more rate acceleration compared to when selenium is acting as a nucleophile relative to sulfur (7). The data also show that sulfur and selenium are comparable when acting as a leaving group.

Here, we have measured the same seleno effect (k_S/k_{Se}) as Koppenol, except that the thiol/disulfide exchange reaction is an enzyme catalyzed reaction instead of a solution phase reaction. We have previously performed an identical experiment with a truncated version of mouse mitochondrial thioredoxin reductase (mTrxR 8) and a truncated thioredoxin reductase from *D. melanogaster* (DmTrxR 8), though we did not interpret the data in terms of a seleno effect (17). We note that mTrxR is a naturally occurring Sec-containing enzyme, while DmTrxR is a Cys-ortholog TrxR that we have used previously for the purpose of direct mechanistic comparisons (15, 17, 18). A summary of seleno effects for mTrxR 8, DmTrxR 8, and PfTrxR 7 is listed in Table 5.

In comparing the summary of reported seleno effects in Table 5 to the model data generated by Koppenol and coworkers in Table 4, a clear pattern emerges. Replacement of the C-terminal Cys residue with Sec results in the smaller ratio of k_S/k_{Se} in comparison to when the N-terminal Cys residue is replaced with Sec. In our study the role of Sec substitution in the thiol/disulfide exchange reaction is limited to either acting as an electrophile or leaving group, unlike the study performed by Koppenol. In determining which of two Cys residues acts an electrophile, we must conclude that it is the C-terminal Cys residue that is in the electrophilic position (subscript “c” in Table 5), based on the magnitude of the seleno effect.

We note in the case of the truncated mammalian enzyme, mTrxR 8, the magnitude of the seleno effect previously reported by us is in very good agreement with the data reported by Koppenol for selenium as an electrophile, and leaving group, respectively. This may be because mTrxR is a naturally occurring Sec-containing enzyme and the thiol/disulfide exchange step that occurs between the N-terminal redox center and the C-terminal redox center may more closely approximate the reaction that occurs in solution. In addition, the reaction does not need to be further enhanced by the enzyme due to the natural rate acceleration that selenium confers to the reaction. Thus, based on interpretation of the seleno effect, it can be concluded that Sec (U) is in the electrophilic position, while the adjacent Cys residue of the Cys-Sec dyad is in the leaving group position (subscript “Lg” in Table 5) in the mammalian enzyme in this exchange step.

For mTrxR⁻⁸ and DmTrxR⁻⁸, we *originally* interpreted the small seleno effect that occurs when Sec replaces the C-terminal Cys residue of the peptide substrate as being due to the superior leaving group ability of a selenolate relative to a thiolate (17). Later, more detailed mechanistic experiments in which we replaced the C-terminal Sec residue of mTrxR and the C-terminal Cys residue of DmTrxR with homocysteine (hCys) led us to reverse this position (15, 18). Our interpretation of the seleno effect reported here supports our later interpretation as selenium acting as the electrophile in the exchange step between N- and C-terminal redox centers.

As mTrxR is a naturally occurring Sec-containing enzyme, the question arises as how Cys-ortholog enzymes accelerate the analogous thiol/disulfide exchange reaction that occurs between N- and C-terminal redox centers. A lack of catalytic enhancement in this step would result in an overall reduction in rate, making Cys-ortholog TrxRs much slower in comparison to the Sec-containing TrxR. This is not experimentally observed as Becker and coworkers have previously shown that the catalytic efficiency of DmTrxR is comparable to the Sec-containing TrxR (16). As shown by Koppenol, selenium accelerates the rate of thiol/disulfide exchange reaction by being both a better nucleophile and better electrophile (7). In the next section we discuss how the electrophilic character of sulfur can be enhanced by the enzyme to accelerate the rate of the thiol/disulfide exchange reaction that occurs between the two redox centers.

Proposed electrophilic activation mechanism

We previously proposed a mechanism by which the enzyme makes the C-terminal disulfide more electrophilic to mimic the electrophilic selenosulfide bond found in the C-terminus of the mammalian enzyme. We termed this mechanism “electrophilic activation” (18). Our proposed electrophilic activation mechanism for Cys-containing TrxRs involves polarization of the C-terminal disulfide through a charged imidazolium cation such that the electrons in the disulfide bond move more closely to the positive charge, rendering the C-terminal sulfur atom (Cys⁵⁴⁰ of PfTrxR) partially electron deficient and hence electrophilic, thereby facilitating the transfer of electrons from the N-terminal thiolate (Figure 5A) (15, 18, 30).

The seleno effect we report for PfTrxR in Tables 1–3 and 5 here provides further, substantial support for this rate acceleration mechanism. While the magnitude of the seleno effect has allowed us to differentiate the roles of each sulfur atom of the Cys⁵³⁵–Cys⁵⁴⁰ disulfide since substitution of Cys⁵⁴⁰ with Sec resulted in an inverse seleno effect of 0.67 and substitution of Cys⁵³⁵ with Sec resulted in a normal seleno effect of 8.7, it also informs that the thiol/disulfide exchange reaction between the two redox centers in the two Cys-TrxRs must be *mechanistically distinct* compared to the Sec-TrxR. This is seen from the data in Table 5 as the magnitude of the seleno effect is very similar for the electrophilic and leaving group sulfur atoms in the two Cys-containing TrxRs, but much different in comparison to the Sec-TrxR.

If the thiol/disulfide exchange reaction between N- and C-terminal redox centers in PfTrxR is accelerated by our proposed electrophilic activation mechanism, then it would be expected that replacement of Cys⁵⁴⁰ with Sec should accelerate the rate due to the enhanced electrophilic character of selenium relative to sulfur (Figure 5B) (6), while the replacement

of Cys⁵³⁵ with Sec is expected to slow the rate (Figure 5C) because the selenolate is considerably less basic than a thiolate (8), and unlike in the solution reaction the selenolate would be in a position where it must interact with the positively charged imidazolium cation.

The 10-fold higher seleno effect observed when Sec is placed in the leaving group position compared to the leaving group seleno effect reported by Koppenol or for mTrxR can be explained by such a mechanism because the protonated cation of the imidazolium ion in effect “solvates” the developing negative charge on the leaving group. In the enzyme catalyzed reaction this interaction between the cation and selenolate of the peptide substrate is fixed because the cation is not mobile, unlike the solution reaction. In the solution reaction, the selenolate could be stabilized by hydrogen bonding to water molecules, a much less unfavorable interaction compared to the “forced” hydrogen bond that must occur between the cationic imidazolium and selenolate. When the leaving group is a thiolate instead, a much more favorable hydrogen bonding interaction could occur in comparison, especially if the pK_a of the thiolate in the transition state is close to the pK_a of the imidazolium cation (32). Alternatively, the thiolate leaving group could be stabilized through an ion pair relationship as has been reported for papain and related enzymes (33, 34).

Our explanation of the larger seleno effect observed in the leaving group position of the thiol/disulfide exchange reaction is analogous to the large thio effects (k_O/k_S) observed in hydrolysis reactions catalyzed by phosphodiesterases when a non-bridging oxygen of the phosphodiester is substituted with sulfur (35–37). Hydrolysis of a phosphodiester results in the build up of negative charge on the non-bridging oxygen atoms in the transition state, which requires stabilization through hydrogen-bonding and charge neutralization. Loss of transition state stabilization occurs upon thio substitution because the negative charge on sulfur is much less stabilized, principally due to a loss of a hydrogen-bonding interaction with the enzyme (35–38). Such a case is illustrated in Figure 6.

The proposed electrophilic activation mechanism for Cys-TrxRs also explains the larger magnitude of the seleno effect when Sec replaces Cys in the electrophilic position compared to the data reported for mTrxR 8 in Table 5 and the data reported by Koppenol summarized in Table 4. Replacement of Cys⁵⁴⁰ with Sec is expected to affect the pK_a of Cys⁵³⁵, which in turn would affect its interaction with His⁵⁰⁹. The alteration of the interaction between Cys⁵³⁵ and His⁵⁰⁹ is expected to slightly increase the electron density around selenium. Therefore the rate acceleration is not expected to be as much as occurs in solution, and this is what is empirically observed.

Further support for the electrophilic activation mechanism by use of homocysteine substitution

As a further test of our proposed electrophilic activation mechanism in PfTrxR, we synthesized peptides **4** and **5**, in which each Cys residue was replaced with hCys. Replacement of either Cys residue with hCys is expected to disrupt the interaction between His⁵⁰⁹ and Cys⁵³⁵ (Figures 5D and 5E), resulting in much less electrophilic activation of the disulfide and lower activity with either peptide substrate. The decrease in activity with either hCys-containing peptide substrate is similar to the loss in activity when Sec replaces Cys⁵³⁵ and is entirely consistent with our proposed mechanism.

Assessing the impact of Sec substitution for Cys⁵³⁵ and Cys⁵⁴⁰

A gain of function that has been previously demonstrated in enzymes in which Sec has replaced Cys is resistance to oxidative inactivation (26, 28). As such, we wanted to test whether the Sec-containing mutants of PfTrxR would also gain this ability, and whether this gain of function would be equivalent at each position. The data show that Cys → Sec substitution at both positions increases resistance to oxidative inactivation by H₂O₂, but a positional affect is clearly evident since the C540U mutant retains most of its activity at 50 mM H₂O₂, but the C535U mutant did not (Figure 4). This type of “seleno effect” with respect to oxidative inactivation is most likely explained by Cys⁵⁴⁰ being in a more solvent exposed position compared to Cys⁵³⁵. This situation would be identical to that of mTrxR, in which the Sec residue is located at the more solvent exposed position compared to the adjacent Cys residue within the C-terminal redox center. The solvent exposed selenolate at position 540 should be in a position to attack H₂O₂, forming a selenenic acid (Sec-SeOH) intermediate. This electrophilic selenenic acid residue is either able to resist further oxidation by being rapidly attacked by Cys⁵³⁵ to form a selenosulfide bond, or potentially be further oxidized to the seleninic acid (Sec-SeO₂⁻). Formation of a seleninic acid would not necessarily result in inactivation of the enzyme, unlike the corresponding sulfinic acid (Cys-SO₂⁻) would in the case of a Cys-containing enzyme. Seleninic acids can be reduced readily by thiols and this may partly explain why Sec-containing enzymes are able to strongly resist inactivation by oxidation (26, 28).

This same rationale explains why the C535U mutant does not resist oxidative inactivation at high concentrations of H₂O₂ in comparison to C540U. In this case, the solvent exposed thiol/thiolate at position 540 is prone to overoxidation to the sulfinic acid and this would result in oxidative inactivation of the mutant enzyme despite the presence of Sec at position 535.

The question that then presents itself is that if the presence of Sec at position 540 in PfTrxR allows for increased resistance to oxidative inactivation and increased catalytic activity as judged by a higher $k_{\text{cat}}/K_{\text{M}}$, why did the enzyme not evolve to contain Sec? There are four selenoproteins coded for by *P. falciparum*, so the organism has the correct biological machinery available to incorporate this amino acid into proteins (42). A possible reason is that the increased hydrogen peroxidase activity that would result from a Cys⁵⁴⁰→Sec substitution could alter the redox balance of the cell, and negatively affect important redox signaling processes that are important to *P. falciparum*'s biology. The answer to this question is not known at present.

CONCLUSIONS

The thio effect ($k_{\text{O}}/k_{\text{S}}$) has been a useful tool for enzymologists to discern information about mechanism of phosphotransferases and phosphohydrolases by comparison of the magnitude of the observed thio effect of the enzymatic reaction to that of known mechanism types that occur in solution. Here, we have introduced an analogous concept, the “seleno effect” ($k_{\text{S}}/k_{\text{Se}}$), which similarly allows for the elucidation of mechanistic information of oxidoreductases that catalyze thiol/disulfide exchange reactions. Comparison of the magnitude of the observed seleno effect in PfTrxR, a cysteine-containing enzyme, has

allowed us to differentiate the role of each Cys residue in the C-terminal disulfide center of the enzyme. Cys⁵³⁵ is in the leaving group position, while Cys⁵⁴⁰ occupies the electrophilic position in the thiol/disulfide exchange reaction that occurs between N- and C-terminal redox centers. The seleno effect also informs that this thiol/disulfide exchange step is significantly different than the same reaction that occurs in the mammalian Sec-containing TrxR. Comparison of the seleno effect in PfTrxR to the mammalian Sec-containing TrxR allows us to conclude that one way Cys-containing TrxRs compensate for the absence of Sec is by making the C-terminal disulfide more electrophilic, accelerating the rate of thiol/disulfide exchange.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The flow of electrons from NADPH to the substrate, Trx, as catalyzed by PfTrxR. (top panel) In the full-length enzyme, FADH₂ reduces the N-terminal redox center, which then undergoes a thiol/disulfide exchange reaction with the C-terminal disulfide (GC⁵³⁵GGGKC⁵⁴⁰G), which in turn reduces Trx. (bottom panel) A peptide (green) with the same sequence as found in the native enzyme can serve as a substrate for the truncated enzyme allowing for the measurement of the rate of thiol/disulfide exchange in this step (X = S or Se).

Figure 2.

Plots of velocity (as mol NADPH/min/mol of PFTxR-7) versus peptide substrate concentration. (A) Enzyme assays of peptides 1 (●), 2 (□) and 3 (■). (B) Enzyme assays of peptides 1 (●), 4 (◇), and 5 (◆). A “seleno effect” is clearly visible in (A) as the peptide in which Cys⁵⁴⁰ is replaced Sec greatly enhances activity (peptide 2), while replacement of Cys⁵³⁵ with Sec diminishes activity (peptide 3).

Figure 3.

Plots of velocity (as mol NADPH/min/mol of PfTrxR $\Delta 7$) versus the concentration of Trx (plot A), or the small molecule substrate selenocystine (plot B). For both plots the WT enzyme is represented by (●), the C540U mutant is represented by (□), and the C535U mutant is represented by (■). As with the truncated enzyme and peptide substrates, a seleno effect is apparent in the full-length Sec-containing enzymes compared to the WT enzyme with both types of substrates used.

Figure 4.

A plot of Trx-reductase activity remaining versus H₂O₂ concentration for WT PfTrxR and Sec-containing mutant enzymes. The blue bars represent the activity of the WT PfTrxR, the red bars represent the activity of the C540U mutant, and the gray bars represent the activity of the C535U mutant.

Figure 5.

Thiol/disulfide exchange reaction between N- and C-terminal redox centers. The C-terminal redox center consists of a β -turn- β motif of sequence GC⁵³⁵GGGKC⁵⁴⁰G. We have previously proposed that this C-terminal disulfide is activated for thiol/disulfide exchange by a mechanism that involves polarization of this disulfide bond via an interaction between a positively charged imidazolium ion, His⁵⁰⁹, and the sulfur of Cys⁵³⁵ (labeled as S_{Lg} for *leaving group*) (30). (A) This polarization results in the electrons of the disulfide bond moving towards the imidazolium ion, resulting in an electron deficient sulfur atom of Cys⁵⁴⁰ (labeled as S_c for *center atom*), activating it for attack by Cys_{IC} (interchange cysteine). (B) When Se replaces Cys⁵⁴⁰, this activation mechanism is intact because the sulfur of Cys⁵³⁵ is still able to hydrogen bond with His⁵⁰⁹. This reaction is further enhanced due to the superior electrophilicity of selenium relative to sulfur (6, 7, 31) and should result in an increase in the rate of thiol/disulfide exchange. (C) When selenium replaces the leaving group sulfur, the interaction between the imidazolium hydrogen bond donor is greatly diminished or completely abolished due to the much worse ability of selenium to act as a hydrogen bond

acceptor relative to sulfur. Such a situation should result in a decreased rate of thiol/disulfide exchange. (D and E) When homocysteine replaces either Cys residue of the disulfide bond, the β -turn- β motif is altered resulting in a disruption of the interaction between the imidazolium cation and the leaving group sulfur. This replacement should result in a decrease in the rate of thiol/disulfide exchange.

Figure 6.

Explanation of the origin of a large non-bridging thio effect observed in some phosphodiesterases. (*left panel*) Attack of a nucleophile onto the phosphorous of a phosphodiester results in development of negative charge on the non-bridging oxygen atoms. The phosphodiesterase stabilizes this negative charge via a hydrogen-bonding interaction (red dash) between a lysine residue and the *pro-S* oxygen. This hydrogen bond could serve to activate the phosphodiester for nucleophilic attack as has been proposed by Frey, Gerlt, and others (38, 39). (*center panel*) When the *pro-R* oxygen atom is replaced with sulfur, a small thio effect occurs because the hydrogen-bonding interaction remains intact. (*right panel*) Replacement of the *pro-S* oxygen atom with sulfur results in a weakened or absent hydrogen bond due to the much weaker tendency of sulfur to act as a hydrogen bond acceptor (40, 41). This results in loss of transition state stabilization and a much larger thio effect ($k_O/k_S' > k_O/k_S$) (35–37). Please note that OR' is assigned higher priority than OR in assigning *pro-R* and *pro-S* designations.

Table 1

Activities of PfTrxR 7 with synthetic peptide substrates.

Peptide	Peptide Sequence	Ring size	k_{cat} (min^{-1})	K_{M} (μM)	V/K^a	Ratio of V/K^b
1	H-G C GGGK C G-OH	20	320 ± 30	4.8 ± 0.9	48.2	—
2	H-G C GGGK U G-OH	20	ND ^c	ND	72.0	0.67
3	H-G U GGGK C G-OH	20	114 ± 32	12 ± 4.6	5.5	8.7
4	H-G C GGGK h C-G-OH	21	ND	ND	NA ^d	NA
5	H-G h CGGGK C G-OH	21	155 ± 12	12 ± 1	NA	NA

^aThe units are mol NADPH/min/mol PfTrxR 7/mM peptide.^bThe ratio of V/K of peptide **1** to the V/K of the selenium-containing peptide is the “seleno effect” for this assay. The method for determining V/K is discussed in the text.^cThese parameters could not be determined from the Michaelis-Menten plot due to a lack of fit.^dThese values are not applicable to the seleno effect.

Table 2Kinetic parameters of Trx-reduction for WT and Sec-containing PfTrxR enzymes.^a

Enzyme	C-terminal sequence	k_{cat} (min ⁻¹)	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ (min ⁻¹ M ⁻¹)	$k_{\text{S}}/k_{\text{Se}}^b$
WT PfTrxR	PfTrxR- C GGGK C G	2120 ± 110	330 ± 25	6.4 × 10 ⁶	—
C540U	PfTrxR- C GGGK U G	561 ± 39	18 ± 5	3.1 × 10 ⁷	0.2
C535U	PfTrxR- U GGGK C G	5.7 ± 0.3	6.6 ± 1.7	8.6 × 10 ⁵	7.4

^aRecombinant *E. coli* thioredoxin is the substrate in this assay.^bRatio of $k_{\text{cat}}/K_{\text{M}}$ of the WT enzyme to $k_{\text{cat}}/K_{\text{M}}$ of the mutant enzyme. This is the “seleno effect” in this assay.

Table 3

Kinetic parameters of selenocystine-reduction for WT and Sec-containing PfTrxR enzymes.

Enzyme	C-terminal sequence	k_{cat} (min^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{min}^{-1} \text{M}^{-1}$)	$k_{\text{S}}/k_{\text{Se}}^a$
WT PfTrxR	PfTrxR- C GGGK C G	990 ± 80	770 ± 90	1.3×10^6	—
C540U	PfTrxR- C GGGK U G	672 ± 73	84 ± 25	8.0×10^6	0.16
C535U	PfTrxR- U GGGK C G	24 ± 1	68 ± 13	3.5×10^5	3.7

^aRatio of $k_{\text{cat}}/K_{\text{M}}$ of the WT enzyme to $k_{\text{cat}}/K_{\text{M}}$ of the mutant enzyme. This is the “seleno effect” in this assay.

Table 4

The seleno effect in model cysteamine/cystamine (selenocystamine) exchange system.

selenium as	nucleophile	electrophile	leaving group
thiol/disulfide exchange reaction ^a
k_S/k_{Se}^b	5.1×10^{-3}	6.9×10^{-5}	9.2×10^{-1}
Relative rate	1.8×10^2	1.33×10^4	1

^aThe electrophilic sulfur or selenium is denoted with subscript “c” (center) and the leaving group sulfur or selenium is denoted with subscript “Lg”.

^bThe average of two measurements reported in (7).

Table 5

Summary of seleno effects for various thioredoxin reductases.

Enzyme	Peptide Substrate ^a	Rate	Rates/Rates _{Se}
mTrxRΔ8 (Sec-TrxR)	H-PTVTGCL _g C _c G-OH	0.03 ^b	—
	H-PTVTGCL _g U _c G-OH*	260 ^b	1.1 x10 ⁻⁴
	H-PTVTGU _{lg} C _c G-OH	0.23 ^b	1.3 x10 ⁻¹
DmTrxRΔ8 (Cys-TrxR)	H-PTPASC _{Lg} C _c S-OH*	41 ^b	—
	H-PTPASC _{Lg} U _c S-OH	499 ^b	8.2 x10 ⁻²
	H-PTPASU _{lg} C _c S-OH	4.6 ^b	8.9
PfTrxRΔ7 (Cys-TrxR)	H-GC _{Lg} GGGKC _c G-OH*	48.2 ^c	—
	H-GC _{Lg} GGGKU _c G-OH	72.0 ^c	6.7 x10 ⁻¹
	H-GU _{lg} GGGKC _c G-OH	5.5 ^c	8.7

^aThe electrophilic position has the subscript “c”, while the leaving group position has the subscript “Lg”.^bRate is reported as mol NADPH/min/mol TrxR/mM peptide as reported in (17).^cRate is reported as mol NADPH/min/mol TrxR/mM peptide in this study.

* Denotes the sequence of the native peptide.