

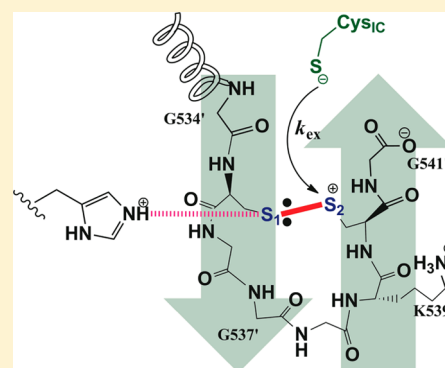
A Mechanistic Investigation of the C-Terminal Redox Motif of Thioredoxin Reductase from *Plasmodium falciparum*

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Supporting Information

ABSTRACT: High-molecular mass thioredoxin reductases (TRs) are pyridine nucleotide disulfide oxidoreductases that catalyze the reduction of the disulfide bond of thioredoxin (Trx). Trx is responsible for reducing multiple protein disulfide targets in the cell. TRs utilize reduced β -nicotinamide adenine dinucleotide phosphate to reduce a bound flavin prosthetic group, which in turn reduces an N-terminal redox center that has the conserved sequence $C_{IC}VNVC_{CT}$, where C_{IC} is denoted as the interchange thiol while the thiol involved in charge-transfer complexation is denoted as C_{CT} . The reduced N-terminal redox center reduces a C-terminal redox center on the opposite subunit of the head-to-tail homodimer, the C-terminal redox center that catalyzes the reduction of the Trx-disulfide. Variations in the amino acid sequence of the C-terminal redox center differentiate high-molecular mass TRs into different types. Type Ia TRs have tetrapeptide C-terminal redox centers of with a GCUG sequence, where U is the rare amino acid selenocysteine (Sec), while the tetrapeptide sequence in type Ib TRs has its Sec residue replaced with a conventional cysteine (Cys) residue and can use small polar amino acids such as serine and threonine in place of the flanking glycine residues. The TR from *Plasmodium falciparum* (PfTR) is similar in structure and mechanism to type Ia and type Ib TRs except that the C-terminal redox center is different in its amino acid sequence. The C-terminal redox center of PfTR has the sequence $G_{534}CGGGKCG_{541}$, and we classify it as a type II high-molecular mass TR. The oxidized type II redox motif will form a 20-membered disulfide ring, whereas the absence of spacer amino acids in the type I motif results in the formation of a rare eight-membered ring. We used site-directed mutagenesis and protein semisynthesis to investigate features of the distinctive type II C-terminal redox motif that help it perform catalysis. Deletion of Gly541 reduces thioredoxin reductase activity by ~ 50 -fold, most likely because of disruption of an important hydrogen bond between the amide NH group of Gly541 and the carbonyl of Gly534 that helps to stabilize the β -turn- β motif. Alterations of the 20-membered disulfide ring either by amino acid deletion or by substitution resulted in impaired catalytic activity. Subtle changes in the ring structure and size caused by using semisynthesis to substitute homocysteine for cysteine also caused significant reductions in catalytic activity, demonstrating the importance of the disulfide ring's geometry in making the C-terminal redox center reactive for thiol-disulfide exchange. The data suggested to us that the transfer of electrons from the N-terminal redox center to the C-terminal redox center may be rate-limiting. We propose that the transfer of electrons from the N-terminal redox center in PfTR to the type II C-terminal disulfide is accelerated by the use of an "electrophilic activation" mechanism. In this mechanism, the type II C-terminal disulfide is polarized, making the sulfur atom of Cys540 electron deficient, highly electrophilic, and activated for thiol-disulfide exchange with the N-terminal redox center. This hypothesis was investigated by constructing chimeric PfTR mutant enzymes containing C-terminal type I sequences GCCG and GCUG, respectively. The PfTR-GCCG chimera had 500-fold less thioredoxin reductase activity than the native enzyme but still reduced selenocystine and lipoic acid efficiently. The PfTR-GCUG chimera had higher catalytic activity than the native enzyme with Trx, selenocystine, and lipoic acid as substrates. The results suggested to us that (i) Sec in the mutant enzyme accelerated the rate of thiol-disulfide exchange between the N- and C-terminal redox centers, (ii) the type II redox center evolved for efficient catalysis utilizing Cys instead of Sec, and (iii) the type II redox center of PfTR is partly responsible for substrate recognition of the cognate PfTrx substrate relative to noncognate thioredoxins.



Thioredoxin reductases (TRs) are homodimeric flavin-containing oxidoreductases that reduce the macromolecular substrate thioredoxin (Trx), thereby serving an important line of antioxidant defense in the cell.¹ Two major classes of TRs are known: (i) low-molecular mass (M_r) TRs (~ 35 kDa per monomer) found in *Escherichia coli* and other lower prokaryotes and (ii) high- M_r TRs (55–60 kDa per monomer) found in higher eukaryotes and *Plasmodium falciparum* as discussed in this

report. Both types of TRs contain a tightly bound FAD and an N-terminal redox center with a CVNVC sequence; however, high- M_r TRs contain a third redox center housed on a C-terminal

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extension of the enzyme, not present in low- M_r TRs.^{2,3} Because of these structural differences, high- and low- M_r TRs have evolved dissimilar catalytic mechanisms for the reduction of Trx.^{4,5}

High- M_r TRs can be classified into two groups based upon different substrate utilization patterns.^{6,7} Type I TRs contain a C-terminal redox center that uses either a cysteine-selenocysteine (Cys–Sec) redox dyad (type Ia) or a Cys–Cys redox dyad (type Ib).⁶ The TR from *P. falciparum* (PfTR) represents a distinct type of high- M_r TR (type II) because instead of using a vicinal arrangement of redox active residues (Cys–Sec or Cys–Cys), it uses a spacer of four residues to separate the two redox active Cys residues for a sequence of G₅₃₄C₁GGGKC₂G₅₄₁.^a To the best of our knowledge, type II TRs have been identified only in apicomplexan protists, including *P. falciparum*,^{8,9} *Cryptosporidium parvum*, and *Toxoplasma gondii*.¹⁰

PfTR is a validated antimalarial drug target because of its requirement for the parasite's survival during the erythrocytic stage of its life cycle.¹¹ This observation and the importance of PfTR to the overall redox biology of the organism have spurred structural and mechanistic investigations into PfTR, with the goal of developing PfTR-targeted antimalarial inhibitors and/or drugs.^{9,12–17} These studies have shown that the type II PfTR has the same general protein architecture as type I TRs, including (i) a head-to-tail arrangement of the homodimer, (ii) a NADPH binding site, (iii) a conserved Rossmann fold that binds FAD, (iv) the same N-terminal redox center C_{CT}VNVGC_{1C},^b (v) a general acid–base His–Glu dyad, (vi) a C-terminal redox center that uses two redox active residues, Cys₁ and Cys₂,^{9,17} and (vii) a mechanism of Trx reduction analogous to that of its type I homologues. The flow of electrons in both types of high- M_r TRs proceeds from NADPH to FAD, then to the N-terminal redox center, followed by transfer to the C-terminal redox center of the opposite subunit of the homodimeric enzyme, and finally to the substrate, Trx. Both Cys residues of the C-terminal redox center are required for effective Trx reduction.⁹ A truncated PfTR missing this C-terminal redox center is still capable of reducing small substrates such as DTNB, in a manner analogous to that of truncated TRs from mouse mitochondria, *Drosophila melanogaster*, and *Caenorhabditis elegans*.^{6,9}

Recently, Becker and co-workers determined the crystal structure of the PfTR–PfTrx complex, providing an insightful glimpse of the structure adopted by the type II C-terminal redox motif.⁷ The C-terminal redox motif of PfTR forms an antiparallel β –turn– β motif that is stabilized by (i) intramolecular hydrogen bonds with His438', (ii) intrastrand interactions, and (iii) intermolecular hydrogen bonds with PfTrx in the PfTR–PfTrx complex (Figure 1A). The structure of the complex shows that a mixed disulfide bond is formed between Cys540' of PfTR^c and Cys30 of PfTrx, providing solid evidence that the C-terminal Cys₂ residue is the nucleophile that attacks PfTrx. This is analogous to human TR in which the Sec residue of the Cys–Sec dyad attacks the disulfide bond of human Trx.¹⁸ While the overall mechanism of PfTR is highly similar to that of type I TRs, little is known about possible catalytic roles served by the bridging (underlined) and flanking amino acids of the C-terminal redox motif (G₅₃₄C₁GGGKC₂G₅₄₁) of PfTR and how this motif functionally differs from the vicinal Cys–Sec and Cys–Cys motif found in type I TRs.

For example, it has been shown that serine (Ser) residues adjacent to the Cys–Cys dyad in the TR from *D. melanogaster* (DmTR) help make the attacking thiolate more nucleophilic by helping to stabilize the negative charge.¹⁹ It is thought that these

adjacent Ser residues help DmTR compensate for the absence of the chemically more reactive Sec residue of the mammalian enzyme. As PfTR lacks Ser residues in its C-terminal redox center, we investigated the hypothesis that the bridging lysine (Lys) residue of the PfTR C-terminal redox center performs a similar function. Another feature of the C-terminal redox center of PfTR is that it will form a 20-membered ring in the oxidized state because of the presence of the four bridging residues between the redox active Cys residues. This redox center differs from the type I TRs that form an eight-membered ring between either adjacent Cys–Sec residues (mammalian TR) or adjacent Cys–Cys residues found in insect TRs from *D. melanogaster* and *Anopheles gambiae*.²⁰

Interestingly, PfTR can reduce PfTrx with an efficiency nearly the same as that with which human TR reduces human Trx,²¹ yet its C-terminal redox center is unique and distinct in comparison. Here, we investigate the features of the C-terminal redox center that allow it to perform efficient catalysis, including (i) the bridging distance between the two redox active Cys residues, (ii) the role of an intrastrand hydrogen bond in stabilizing the β –turn– β motif, and (iii) the precise shape of the 20-membered ring by the use of homocysteine (hCys) for Cys substitution. In addition, we show that PfTR can still function after its type II redox center has been replaced with a type I motif if the nucleophilic Cys₂ residue is replaced with Sec. The findings presented in this report reveal novel mechanistic and functional characteristics of PfTR's C-terminal redox center that demonstrate the dependence on the native type II C-terminal redox motif in conducting the reduction of Trx, but not other small molecule substrates such as selenocystine and lipoic acid.

MATERIALS AND METHODS

Materials. All mutagenic polymerase chain reaction (PCR) primers utilized in the construction of PfTR mutants were purchased from Integrated DNA Technologies (Coralville, IA). The Quickchange site-directed mutagenesis kit was purchased from Agilent Technologies (Santa Clara, CA). Vent DNA polymerase, NdeI, SapI, T4 DNA ligase, *E. coli* ER2566 competent cells, and chitin-agarose beads were purchased from New England Biolabs (Ipswich, MA). GSH-sepharose 4 Fast Flow, benzamidine sepharose, and 2'-4'-ADP-sepharose resins were obtained from GE Healthcare (Waukesha, WI). Sephacryl S-200 resin was purchased from Amersham Pharmacia (Uppsala, Sweden). NADPH was obtained from AppliChem (Darmstadt, Germany). *N*-(Methyl)mercaptoacetamide, β ME, and DL- α -lipoic were from Sigma-Aldrich (St. Louis, MO). Bovine thrombin was purchased from BioPharm Laboratories (Bluffdale, UT). L-Selenocystine was from Acros Organics (Morris Plains, NJ). Hydrogen peroxide (30% solution) and EDTA were purchased from Fisher Scientific (Fair Lawn, NJ). All enzyme kinetic assays were performed on a Cary50 UV–vis spectrophotometer (Cary, Walnut Creek, CA) and were conducted at room temperature.

Site-Directed Mutagenesis To Generate Fully Recombinant PfTR Mutants. All enzymes in this study are numbered 1–13 as described in Tables 1–4. Wild-type (WT) PfTR (enzyme 1) was a gift from F. Angellucci of the University of Rome (Rome, Italy) and is produced as a glutathione S-transferase fusion protein (GST fusion) from a pGEX-4T-1 plasmid as described in the next section. Enzymes 3–8 and 11 were generated via site-directed mutagenesis of the plasmid bearing the WT coding sequence utilizing the Quickchange II kit from Agilent Technologies. The plasmids containing the desired mutations were constructed by PCR amplification using an

Eppendorf Mastercycler PCR system. Mutagenesis was conducted with the *Pfu* Ultra DNA polymerase (Agilent Technologies) using 18 cycles under the following parameters: 95 °C for 50 s, 60 °C for 60 s, and 68 °C for 14 min. The remainder of the site-directed mutagenesis procedure was followed as per the manufacturer's instructions. All of the mutants were sequenced by the University of Vermont DNA Sequencing Facility (Burlington, VT) using an ABI 3100-Avant genetic analyzer.

Purification of PfTRs Expressed as GST Fusion Constructs.

Enzymes 1, 3–8, and 11 were expressed in ER2566 *E. coli* cells (New England Biolabs) and grown in Terrific broth medium with supplementation with riboflavin, niacinamide, and pyridoxine (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 expression was induced by the addition of IPTG to a final concentration of 0.5 mM with subsequent incubation overnight at 20 °C with shaking. After overnight incubation, the cells were harvested by centrifugation (9000 rpm in a Beckman J2-21 preparatory centrifuge for 10 min at 4 °C) and either used immediately or frozen at –20 °C for later use. The harvested cell pellet was homogenized in buffer A [50 mM Tris-HCl, 200 mM NaCl, and 2 mM β-mercaptoethanol (βME) (pH 7.4)] with the inclusion of 1 mg/mL chicken egg lysozyme to aid in the lysis of bacterial cell walls. The homogenized cells were further lysed by probe sonication, and the solution was cleared by centrifugation at 12000 rpm in a Beckman J2-21 preparatory centrifuge for 1 h at 4 °C. The cleared lysate was loaded by gravity onto a column packed with glutathione sepharose 4 fast flow resin (GE Healthcare) that had been pre-equilibrated with buffer A. The loaded column was washed extensively with buffer A followed by additional washing with buffer B [50 mM Tris-HCl, 500 mM NaCl, and 2 mM βME (pH 7.4)]. After being washed, the column was rendered as a slurry mix and transferred to two 50 mL conical tubes. Cleavage of the fusion protein was initiated by addition of 500 U.S. units of bovine thrombin supplemented with 5 mM CaCl₂ to the resin/protein slurry. The thrombin-mediated cleavage of PfTR from the GST fusion protein proceeded overnight at room temperature with constant agitation. After overnight incubation, the protein/resin slurry was returned to the column and the liberated PfTR enzyme was eluted off the resin by washing with buffer B. Fractions were analyzed by UV-vis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and positive fractions were pooled and loaded onto a benzamidine sepharose column (GE Healthcare) that had been pre-equilibrated with 50 mM Tris-HCl, 500 mM NaCl buffer (pH 8.0). Under these conditions, benzamidine sepharose binds thrombin with high affinity but does not bind PfTR. This chromatographic step eliminates residual thrombin from the sample. Following elution off the benzamidine column, fractions containing PfTR were pooled and dialyzed overnight against a final storage buffer [50 mM potassium phosphate, 500 mM NaCl, and 1 mM EDTA (pH 8.0)]. The PfTR enzyme was judged to be pure and free of thrombin by SDS–PAGE analysis (shown in Figure S1 of the Supporting Information).

Production and Purification of Semisynthetic PfTR Mutants. The production of semisynthetic PfTR mutant constructs 9, 10, 12, and 13 was achieved via intein-mediated peptide ligation²² in a manner analogous to that previously described in our work with semisynthetic TRs from mouse mitochondria and *D. melanogaster*.^{23,24} A truncated construct lacking the final seven C-terminal amino acids (PfTRΔ7) was fused to the *See* VMA intein as provided by New England Biolabs to

produce a PfTRΔ7–intein fusion protein. The PfTRΔ7 construct was generated through deletion of the final seven C-terminal amino acids via PCR amplification of the full length WT PfTR plasmid DNA template. The following primers were used: upstream primer 5'-AACAGACATATGGGATCCTGC-AAA-3', which introduced a NdeI restriction site, and downstream primer 5'-ACAGCCGCTCTTCAGCAACCACCTTT-3', which contained the seven-amino acid deletion, the insertion of a Cys residue (required for thiol-mediated liberation from the intein), and a SapI endonuclease restriction site. PCR was performed on an Eppendorf Mastercycler using Vent DNA polymerase. The amplified DNA was then digested with NdeI and SapI and shuttled into the pTYB1 expression vector (New England Biolabs) with subsequent ligation conducted by T4 DNA ligase. Sequencing of the PfTRΔ7 mutant was performed at the University of Vermont DNA Sequencing Facility using an ABI 3100-Avant genetic analyzer.

The semisynthetic PfTR enzymes were produced via ligation of desired synthetic peptides to the truncated PfTRΔ7 containing a C-terminal thioester reactive group. Peptides containing an N-terminal Cys residue can be ligated (to form an amide bond) to peptides containing a C-terminal thioester.²² The thioester-tagged PfTRΔ7 protein is produced initially as a PfTRΔ7–intein fusion protein expressed as a recombinant protein in ER2566 *E. coli* cells using growth conditions identical to those described above for the PfTR–GST fusion protein. The harvested bacterial cell pellet was homogenized in chitin buffer A [50 mM MOPS and 150 mM NaCl (pH 7.0)] followed by cell lysis via pulse sonication. The lysed cell slurry was 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 loaded PfTR protein was further washed with 0.5 L of chitin buffer A followed by additional washing with 0.5 L of high-salt chitin buffer B [50 mM MOPS and 500 mM NaCl (pH 7.0)]. The resin containing the bound protein was then converted to a slurry by the addition of chitin buffer B and then transferred to two 50 mL conical tubes. Simultaneous cleavage of the fusion protein and ligation with synthetic peptide were achieved by addition of a cleavage cocktail consisting of 120 mM *N*-methyl mercaptoacetamide (NMA) and the desired synthetic peptide in chitin buffer B (pH 8.0–8.5). The cleavage and ligation reaction proceeded overnight at room temperature with constant agitation. Following overnight incubation, the chitin/resin slurry was returned to the column and the liberated semisynthetic enzyme was eluted via gravity flow by further washing of the resin with chitin buffer B. The semisynthetic enzyme was concentrated and buffer exchanged with buffer C [10 mM Tris-EDTA, 10 mM NaCl, and 20 mM βME (pH 8.0)] via ultrafiltration (Amicon Ultracel 30 kDa cutoff filters). The semisynthetic enzyme was then loaded by gravity onto a pre-equilibrated 2',4'-ADP-sepharose column (20 mL). After the sample had been extensively washed with buffer C, the semisynthetic PfTR enzyme was eluted off the 2',4'-ADP-sepharose column with high-salt buffer [10 mM Tris-EDTA, 1 M NaCl, and 20 mM βME (pH 8.0)]. The PfTR-positive fractions were confirmed by spectrophotometric and SDS–PAGE analyses, pooled, and then extensively buffer exchanged by ultrafiltration with storage buffer [50 mM potassium phosphate, 500 mM NaCl, and 1 mM EDTA (pH 8.0)]. The concentration of each PfTR semisynthetic enzyme was determined spectrophotometrically via the absorbance maximum of the flavin at 460 nm ($\epsilon = 22.6 \text{ mM}^{-1} \text{ cm}^{-1}$, representing the dimer).

The truncated enzyme (enzyme 2) was produced for use in kinetic studies by cleaving the PfTR Δ 7–intein fusion protein in the presence of NMA and the absence of peptide. After cleavage from the chitin–agarose column, pooled fractions of enzyme 2 were concentrated by ultrafiltration and loaded onto a Sephacryl-S200 column (size exclusion) that had been pre-equilibrated with 50 mM potassium phosphate, 500 mM NaCl, and 1 mM EDTA (pH 8.0). TR-positive fractions eluted off the Sephacryl-S200 column and were then pooled and concentrated.

Peptides with amino acid sequences of H-hCGGGKCG-OH and H-CGGGKhCG-OH were used in the construction of enzymes 9 and 10, respectively. These peptides were produced by standard Fmoc solid phase synthesis protocols as described by us previously.²³ Peptides with amino acid sequences of H-CUG-OH and H-CGGGKUG-OH were used to construct enzymes 12 and 13, respectively (U is the one-letter abbreviation for selenocysteine). The protected amino acid precursor Fmoc-Sec(Mob)-OH was used for the syntheses of these selenium-containing peptides also using standard Fmoc protocols as described by us previously.²³ The Mob (4-methoxybenzyl) group was removed by using an innovative protocol using dithionitropyridine as a deprotection reagent described by us for the simultaneous cleavage from solid phase supports and removal of the protecting Mob group.^{25,26}

Activity Assays with Thioredoxin as the Substrate. All PfTR enzymes except the truncated enzyme were assayed for their ability to reduce *E. coli* Trx as previously described.²⁷ Briefly, the 0.5 mL reaction mixture contained 150 μ M NADPH, 1 mM EDTA, 90 μ M *E. coli* Trx, and 0.16 mM bovine pancreas insulin in 50 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of PfTR, and the activity was monitored by the decrease in absorbance at 340 nm (A_{340}) attributed to the consumption of NADPH. The concentration of each respective PfTR construct in the Trx reductase assay is listed in Table S1 of the Supporting Information. Enzymes 1, 12, and 13 were also assayed under conditions identical to those described above, but using varying concentrations of Trx to determine the kinetic parameters k_{cat} and K_m . All kinetic assays (with Trx, selenocystine, and lipoic acid as substrates) were conducted at room temperature. For this assay and all similar enzyme assays, we corrected for background NADPH consumption by subtracting the activity of control experiments in which either TR or the substrate was omitted from the reaction mixture. The assays were repeated in triplicate for each enzyme.

Activity Assays with Selenocystine as the Substrate. All PfTR enzymes in this study were also assayed for their ability to catalyze the reduction of the small molecule diselenide, selenocystine, as previously described.²⁸ The 1 mL reaction mixture contained 10 mM EDTA, 200 μ M NADPH, and 300 μ M selenocystine in 500 mM potassium phosphate (pH 7.0). The reaction was initiated by the addition of the PfTR enzyme, and the activity was monitored by following the decrease in A_{340} . The concentration of each PfTR construct in the selenocystine reductase assay is listed in Table S2 of the Supporting Information.

Activity Assays with Lipoic Acid as the Substrate. All PfTR enzymes in this study were also assayed for their ability to reduce the small molecule substrate lipoic acid. The 0.5 mL reaction mixture contained 1 mM EDTA, 150 μ M NADPH, 5 mM α -DL-lipoic acid substrate, and 50 nM TR enzyme in 500 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of TR, and the activity was monitored by the decrease in A_{340} .

Activity Assays with Hydrogen Peroxide as the Substrate. Enzymes 1, 12, and 13 were assayed for their abilities to catalyze the reduction of H_2O_2 , as previously described.²⁹ Stock solutions of H_2O_2 were freshly prepared before each experiment with the concentration of H_2O_2 determined spectrophotometrically using an extinction coefficient of $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ at 240 nm.³⁰ The 0.5 mL reaction mixture consisted of 200 μ M NADPH, 2 mM EDTA, and 50 mM H_2O_2 in 100 mM potassium phosphate buffer (pH 7.0). The concentrations of TR in the peroxidase assays were as follows: 400 nM for enzyme 1, 30 nM for enzyme 12, and 30 nM for enzyme 13. The activity was measured by monitoring the decrease in A_{340} .

RESULTS AND DISCUSSION

Features of the β -Turn- β Motif That Contribute to Catalysis. We initially explored the hypothesis that Lys539' acted as a type of acid–base catalyst to help increase the nucleophilic character of Cys540'. Our activity assays depicted in Table 1 show that the enzyme loses only a small amount of

Table 1. Activities of WT PfTR and Mutant Enzymes of the C-Terminal β -Turn- β Motif with Various Substrates^a

enzyme	PfTR	mol of NADPH min ⁻¹ (mol of TR) ⁻¹		
		90 μ M Trx	300 μ M selenocystine	5 mM lipoic acid
PfTR-GC ₁ GGGKC ₂ G ^b	1	500 \pm 20	410 \pm 30	90 \pm 10
PfTR Δ 7 ^c	2	ND ^d	3 \pm 0.2	140 \pm 1
PfTR-GC ₁ GGGAC ₂ G ^b	3	375 \pm 20	350 \pm 15	65 \pm 5
PfTR-GC ₁ GGGKC ₂ ^b	4	7 \pm 0.5	185 \pm 10	70 \pm 3
PfTR-GC ₁ GGGKC ₂ GG ^b	5	290 \pm 10	400 \pm 20	90 \pm 2

^aIn Tables 1–4, the main body of the enzyme is abbreviated as PfTR. The amino acid sequence of the C-terminal redox center is abbreviated with the one-letter amino acid codes representing the final seven C-terminal amino acids. Thus, the WT enzyme is abbreviated as PfTR-GC₁GGGKC₂G. Each of the mutants is denoted similarly with the mutant sequence of amino acids of the C-terminal redox center abbreviated with one-letter codes after the abbreviation PfTR. ^bRecombinant protein produced as a GST fusion. ^cRecombinant protein produced as an intein fusion. ^dNo detectable activity.

activity toward both *E. coli* Trx and small molecule substrates when this Lys residue is mutated to Ala (enzyme 3), eliminating any important acid–base catalysis role of Lys539'. The crystal structure of the PfTR–PfTrx complex shows the Lys539' ϵ -amino group interacting with Ser71 of PfTrx, but this interaction must make only a very small contribution to substrate binding based on our data.

Next, we investigated the role of the C-terminal Gly541' residue in catalysis. Structural studies of other high- M_r TRs suggested that a salt bridge between the carboxylate of Gly541' and Lys58 serves as an anchor for reduction of the C-terminal disulfide.^{7,31} In PfTR, it was proposed that a salt bridge between the carboxylate of Gly541' and Lys58 serves as this anchor function.⁷ The crystal structure of the PfTR–PfTrx complex also shows that there is a hydrogen bond between the amide NH group of Gly541' and the carbonyl of Gly534' as highlighted in Figure 1A. Deletion of this C-terminal Gly residue (enzyme 4) has a very large effect on Trx reductase activity, but only a small effect on the selenocystine reductase activity or on the reduction of lipoic acid. Addition of an extra Gly residue to the β -turn- β motif (enzyme 5), which would eliminate the proposed salt bridge (see above), yields almost the same activity as WT.

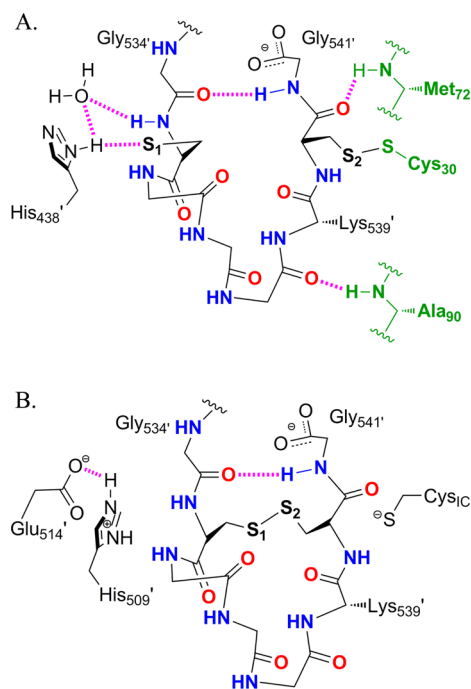


Figure 1. (A) C-Terminal redox center of PfTR in complex with PfTrx. This figure is adapted from Figure 2 of ref 7. The β -turn- β motif is shown with carbonyl oxygen atoms colored red and amide nitrogen atoms blue. Residues from PfTrx are colored green. Hydrogen bonds are shown as dashed lines (magenta). A key stabilizing intrastrand interaction is the hydrogen bond between the NH group of Gly541' and the carbonyl of Gly534'. The sulfur atoms of Cys535' and Cys540' are labeled as S₁ and S₂, respectively (bold black). In this "out" position of the β -turn- β motif, Cys540' is involved in a mixed disulfide bond with Cys30 of PfTrx in the complex.⁷ The His438'-water dyad is proposed to act as an acid-base catalyst to deprotonate S₁ of Cys535' so that this thiolate can resolve the mixed disulfide bond of the complex.⁷ This interaction is shown here as a hydrogen bond between S₁ and His438'. (B) Here the β -turn- β motif is shown in the "in" position. After Cys535' resolves the mixed disulfide bond of the complex, a disulfide bond is formed between Cys535' and Cys540'. The β -turn- β motif then moves 20 Å toward the interior of the enzyme so that this C-terminal redox center can be reduced by the N-terminal redox center. Shown here are the interchange Cys residue (Cys_{IC}) and the His509'-Glu514' dyad that is known to be the general acid-base catalyst for this thiol-disulfide exchange reaction. His509' is important for our proposed electrophilic activation mechanism.

We interpret this to mean that the hydrogen bond between the amide of Gly541' and the carbonyl of Gly534' is more important for stabilizing this β -turn- β motif relative to the proposed anchor function of Gly541'. The stabilization via the hydrogen bond between Gly541' and Gly534' is most likely important

both for the reduction of Trx and for the reduction of the disulfide of the C-terminal redox center.

Assessing the Importance of Disulfide Ring Size of the β -Turn- β Motif to Catalysis. The type II C-terminal redox center differs from type I redox centers in both total length and size of the disulfide ring formed upon oxidation of the two C-terminal Cys residues. Type I TRs form a disulfide bond between adjacent residues, which is a rare occurrence for proteins listed in the Protein Data Bank.³² The C-terminal redox center of PfTR forms a much more commonly used 20-membered ring, just as is found in the N-terminal redox center of the enzyme. It is likely that the bridging distance between the two redox active Cys residues and optimal tail flexibility favor catalytically productive binding and orientation of PfTrx. The presence of three consecutive Gly residues, which contain a high degree of conformational freedom, lends support to this hypothesis. In addition, the C-terminal disulfide bond of PfTR that results from the reduction of PfTrx is itself a substrate for the N-terminal redox center. The precise geometry of this 20-membered disulfide most likely causes it to be a reactive disulfide, making it easier to be reduced by the N-terminal redox center.

Mutant enzymes 6–8 were constructed to investigate these ideas. The data in Table 2 show that decreasing the ring size to 17 atoms in enzymes 6 and 7 greatly impacts the reduction of Trx, while the reduction of both selenocystine and lipoic acid is much less effected. While both mutants are deletions of one residue between the two redox active Cys residues, the Trx reductase activity of enzyme 6 (deletion of Lys) is more significant, being reduced by ~1000-fold versus a 6-fold smaller activity for the loss of one glycine.

The addition of one residue between Cys535' and Cys540' would potentially result in a disulfide ring of 23 atoms (enzyme 8), assuming the ring actually forms in the mutant. This addition results in a 50-fold decrease in Trx reductase activity. Even very subtle changes in disulfide ring size result in a large loss of activity as shown by the substitution of homocysteine (hCys) for Cys in mutant enzymes 9 and 10. The side chain of hCys is one methylene unit longer (3 Å) than Cys; either this extra length must affect the nucleophilic attack of thiolate on the disulfide bond of Trx, or the resulting 21-membered disulfide ring is a poor substrate for the N-terminal redox center (in comparison to the 20-membered disulfide ring in the WT enzyme). The low Trx reductase activities of enzymes 9 and 10 cannot be due to poor ligation of the synthetic peptide to PfTR Δ 7, as evidenced by the fact these mutants possess significant selenocystine reductase activity. We have proven the selenocystine substrate cannot be efficiently reduced by TRs lacking an intact C-terminal redox center (see truncated enzyme 2 in Table 1); thus, the selenocystine reductase assay can be used as a functional assay to conduct a semiquantitative assessment of ligation efficiency.^{6,28} If the peptide were not ligated to the enzyme, the mutants would have

Table 2. Activities of PfTR Mutant Enzymes That Have C-Terminal Disulfide Ring Sizes with Various Substrates

enzyme	PfTR	ring size	mol of NADPH min ⁻¹ (mol of TR) ⁻¹		
			90 μ M Trx	300 μ M selenocystine	5 mM lipoic acid
PfTR-GC ₁ GGGKC ₂ G ^a	1	20	500 \pm 20	410 \pm 30	90 \pm 10
PfTR-GC ₁ GGGC ₂ G ^a	6	17	0.4 \pm 0.3	60 \pm 3	75 \pm 5
PfTR-GC ₁ GGKC ₂ G ^a	7	17	8 \pm 1	190 \pm 10	125 \pm 5
PfTR-GC ₁ GGGGKC ₂ G ^a	8	23	10 \pm 1	340 \pm 20	100 \pm 5
PfTR-GhC ₁ GGGKC ₂ G ^b	9	21	20 \pm 1	190 \pm 10	100 \pm 5
PfTR-GC ₁ GGGKhC ₂ G ^b	10	21	3 \pm 0.3	150 \pm 6	140 \pm 6.5

^aRecombinant protein produced as a GST fusion. ^bRecombinant protein produced as an intein fusion via semisynthesis.

Table 3. Activities of the PfTR Chimeric Mutants with Trx, Selenocystine, and Lipoic Acid

enzyme	PfTR	mol of NADPH min ⁻¹ (mol of TR) ⁻¹			
		90 μM Trx	300 μM selenocystine	5 mM lipoic acid	50 mM H ₂ O ₂
PfTR-GC ₁ GGGKC ₂ G ^a	1	500 ± 20	410 ± 30	90 ± 10	9 ± 0.2
PfTR-GC ₁ C ₂ G ^a	11	1.3 ± 0.6	280 ± 15	70 ± 2	ND ^c
PfTR-GC ₁ U ₂ G ^b	12	1160 ± 20	2130 ± 60	255 ± 10	330 ± 3
PfTR-GC ₁ GGGKU ₂ G ^b	13	85 ± 5	260 ± 15	145 ± 5	60 ± 3

^aRecombinant protein produced as a GST fusion. ^bRecombinant protein produced as an intein fusion via semisynthesis. ^cNo activity detectable.

selenocystine reductase activity similar to that of the truncated enzyme (enzyme 2).

For all of the mutants listed in Tables 1 and 2, there is very little effect on the reduction of lipoic acid because, as we have previously shown, the N-terminal redox center of PfTR is sufficient to reduce this disulfide.⁶ High-*M_r* TRs are structurally related to both glutathione reductase and lipoamide dehydrogenase,² but high-*M_r* TRs have a C-terminal extension containing the C-terminal redox center that is functionally equivalent to oxidized glutathione and lipoic acid. Thus, the truncated PfTR can be considered a “mutant” lipoamide dehydrogenase capable of reducing lipoic acid. We note that while the truncated PfTR will reduce lipoic acid, it will not reduce oxidized glutathione. The disulfide bond of lipoic acid is much more strained (electrophilic) in comparison to the disulfide bond of oxidized glutathione, and this supports our proposed “electrophilic activation” mechanism discussed below. In the case of selenocystine as the substrate, the enzyme does not need to use both Cys535' and Cys540' to reduce the diselenide and can use a mechanism in which only one of these residues is present.⁶ This means that the thiolate of either Cys535' or Cys540' is capable of attacking the substrate diselenide, and that a mixed selenosulfide between PfTR and Sec is capable of being reduced by the N-terminal redox center. In other words, the PfTR can use a mechanism that does not involve formation of a disulfide between Cys535' and Cys540' to reduce the diselenide of selenocystine.⁶

Effect of Sec Substitution on Mutant PfTR Chimeric Enzymes. The results given above showed that tail length and disulfide ring geometry are unique features of the type II C-terminal redox center. However, the results did not provide much insight into how PfTR can catalyze the reduction of *E. coli* Trx with a high *k_{cat}* value (~2100 min⁻¹) without the need for Sec in comparison to the Sec-dependent mammalian enzyme, which catalyzes the reduction of *E. coli* Trx with a *k_{cat}* of 3300 min⁻¹.³³

To address this question, we constructed a chimeric enzyme in which we replaced the type II C-terminal redox center of PfTR with that of a type I redox center (enzyme 11) like that found in the mitochondrial TR from *C. elegans*.³⁴ Not surprisingly, this chimeric enzyme had very little Trx reductase activity but was still able to reduce both selenocystine and lipoic acid efficiently (Table 3). However, the Trx reductase activity of enzyme 12, which has Sec in place of Cys in the second position of the redox center, had activities with all three substrates higher than that of the WT enzyme.

Here is an apparent example of the “catalytic superiority” of Sec compared to Cys as has been previously demonstrated by others.³⁵ Given this superiority, why did PfTR not evolve the use of Sec in its C-terminus? *P. falciparum* has four selenoproteins and thus contains all of the necessary components of the Sec insertion machinery needed to accomplish this change in PfTR.^{36,37} We suggest three reasons. First, as shown in Table 3, the Sec for Cys substitution (enzyme 12) results in a hydrogen peroxidase activity ~33-fold higher than that of the WT enzyme.

This gain of function might negatively affect the redox biology of *P. falciparum* in some way. Second, the unique type II C-terminal redox motif may provide PfTR with a means of discriminating between its cognate PfTrx substrates and those found in the vector (*A. gambiae* Trx) or human (hTrx) hosts encountered throughout its life cycle. Third, the type II motif may increase catalytic efficiency with its cognate PfTrx substrate without using Sec. Indeed, the PfTR enzyme displays increased catalytic efficiency with PfTrx (*K_m* = 2.1 μM; *k_{cat}* = 1674 min⁻¹; *k_{cat}*/*K_m* = 791) compared to that of *E. coli* Trx (*K_m* = 500 μM; *k_{cat}* = 1688 min⁻¹; *k_{cat}*/*K_m* = 3),¹⁶ a relationship also observed with high-*M_r* TR orthologs from other species.²¹

Support for this last idea is given by the data presented in Table 4, which lists the kinetic constants *k_{cat}* and *K_m* with *E. coli*

Table 4. Kinetic Constants of the Trx Reductase Activity of WT PfTR and Mutant Enzymes^a

enzyme	PfTR	<i>k_{cat}</i> (min ⁻¹)	<i>K_m</i> (μM)
PfTR-GC ₁ GGGKC ₂ G ^b	1	2120 ± 110	330 ± 25
PfTR-GC ₁ U ₂ G ^c	12	1440 ± 50	20 ± 3
PfTR-GC ₁ GGGKU ₂ G ^c	13	120 ± 8	20 ± 5

^a*E. coli* Trx was used as the substrate. ^bRecombinant protein produced as a GST fusion. ^cRecombinant protein produced as an intein fusion via semisynthesis.

Trx as the substrate for the WT and Sec-containing mutants. The use of Sec in both mutant enzymes has the effect of significantly decreasing the *K_m* for *E. coli* Trx. The dramatic decrease of the *K_m* means a higher affinity for *E. coli* Trx, as can be visually seen in the Michaelis–Menten plots for these assays (displayed in Figure S2 of the Supporting Information).

We also made the same Sec for Cys substitution using semisynthesis in the context of the native type II motif (enzyme 13). The results show that it has Trx reductase activity lower than that of the WT enzyme (Table 3). The low activity may be due to incomplete ligation of the peptide to the truncated enzyme. We base this conclusion on the rather low selenocystine reductase activity of enzyme 13 compared to that of enzyme 12 (Table 3). We would expect that both PfTR Sec enzymes would have similar selenocystine reductase activity because of the presence of both Sec and a C-terminal Cys residue in each construct. Thus, this discrepancy in selenocystine reductase activities leads us to assume enzyme 13 would have Trx reductase activity very similar to (or higher than) that of the WT enzyme.

Catalytic Effect of Insertion of Sec into PfTR and Implications for the Catalytic Mechanism of PfTR. What is the chemical basis for the high activity seen in the Sec-containing chimeric mutant (enzyme 12), and what does that mean for the catalytic mechanism of PfTR? We can conclude that the use of Sec in the chimeric enzyme permits electrons to flow efficiently from the N-terminal redox center to the Sec-containing redox center, and then finally to Trx. This same flow of electrons is

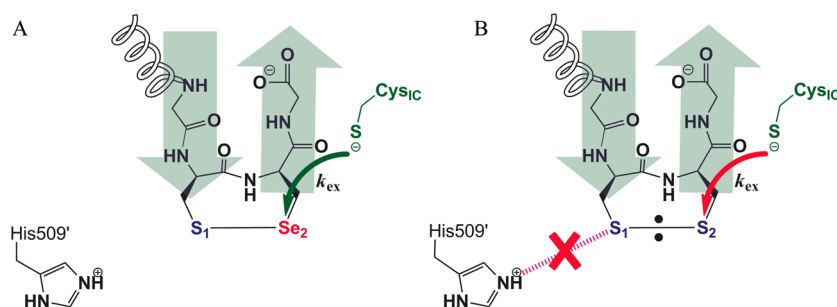


Figure 2. Explanation of the high activity with Sec that is observed in chimeric PfTR 12. (A) The chimeric PfTR enzyme 12, containing a type I C-terminal redox center with a Cys₁–Sec₂ dyad, has high activity because (i) it can attack the disulfide bond of Trx efficiently because the selenolate is a good nucleophile and (ii) the selenium atom of the selenosulfide bond is a good electrophile and accelerates the flow of electrons from the N-terminal redox center to the C-terminal redox center. (B) When the selenium atom is replaced with sulfur as in chimeric enzyme 11, there is little activity for the opposite reasons stated above: the thiolate is a weaker nucleophile, and the Cys₁–Cys₂ disulfide bond of the mutant C-terminal redox center is a weaker electrophile (in this context), and this inhibits the flow of electrons from the N-terminal redox center to the C-terminal disulfide. As depicted here, this is because the disulfide bond is not polarized (even sharing of electrons) as much as the S–Se bond.

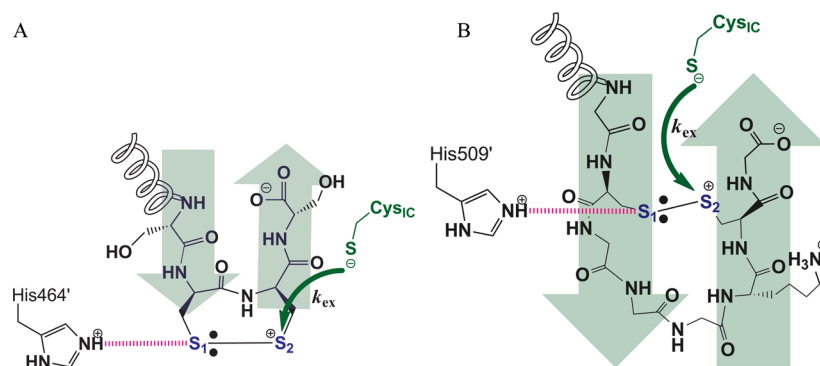


Figure 3. Explanation of the proposed electrophilic activation mechanism for Cys TR enzymes. (A) In DmTR, a type I insect TR that naturally contains a Cys₁–Cys₂ dyad, we have proposed that the reason that electrons can be easily transferred to the C-terminal disulfide is due to a high degree of polarization in the disulfide bond by an adjacent HisH⁺ residue (electrophilic activation mechanism).⁴⁰ (B) The flow of electrons from the N-terminal redox center of PfTR could also be facilitated by a similar electrophilic activation mechanism. His509' could serve to polarize the disulfide bond of the GC₁GGGKC₂G redox motif as shown here.

obviously impaired in the Cys-containing chimera (enzyme 11). The high activity seen in enzyme 12 could be due to the ability of Sec to more efficiently attack the disulfide bond of Trx (Sec as a good nucleophile),³⁸ or it could be that the selenium atom of a selenosulfide is a better acceptor of electrons from Cys_{IC} than is the sulfur atom of a disulfide (selenide as a good electrophile)³⁹ in the context of the same C-terminal redox motif (Gly-Cys₁-Sec₂-Gly vs Gly-Cys₁-Cys₂-Gly). Please see panels A and B of Figure 2 for a comparison and further explanation.

Analyses of the mutants containing hCys in place of Cys (enzymes 9 and 10) are useful for clarifying the two roles just mentioned. Both of these mutants have activity much lower than that of the WT enzyme. It seems reasonable to assume that the sulfur atoms of Cys₂ and hCys₂ have the same nucleophilic reactivity, and thus, enzymes 1 and 10 are expected to be able to attack the disulfide bond of Trx equally well. However, we cannot exclude the possibility that the pK_a of the hCys residue at position 540' is greatly perturbed relative to that of Cys at the same position, and the loss of nucleophilic character of this thiolate in the ES complex greatly reduces the rate.

An alternative possibility that we have recently come to favor is that reduction of the C-terminal Cys-hCys disulfide in the mutant is impaired and this results in the observed low rate of Trx reduction. This interpretation means that the observed difference in activity between enzymes 1 and 10 is due to the inability of the C-terminal redox center of enzyme 10 (containing hCys₂) to

accept electrons from Cys_{IC}. This would be due to a lack of electrophilic activation of the disulfide bond. Similarly, we recently proposed that the C-terminal redox center of DmTR containing a C-terminal Ser-Cys₁-Cys₂-Ser disulfide bond is positioned optimally to be activated for thiol–disulfide exchange because of polarization of the disulfide bond by His464' (equivalent to His509'), as explained in the legend of Figure 3A.⁴⁰ We therefore hypothesize that PfTR uses a similar electrophilic activation mechanism for efficient reduction of its type II C-terminal disulfide as explained in the legend of Figure 3B.

This putative activation interaction described above would occur in the so-called “in” conformation, when the C-terminal tail is closest to the N-terminal redox center and neighboring His509'–Glu514' dyad (shown in Figure 1B), as described by McMillan and colleagues.¹⁷ Unfortunately, this conformation was not captured in the PfTR–PfTrx cocrystal structure, which instead featured the mixed disulfide between PfTR and PfTrx that occurs in the “out” conformation, with the type II C-terminal redox center being ~20 Å from the N-terminal redox center, so that the C-terminal redox center is now positioned close to the His438'–water dyad as shown in Figure 1A.⁷

The C-terminal disulfide could be activated (made more electrophilic) in a manner different from our proposed electrophilic activation mechanism. An alternative is that the 20-membered disulfide ring is strained in the “in” conformation and the relief of strain drives the thiol–disulfide exchange

reaction that occurs between the N- and C-terminal redox centers. We suggest that the hydrogen bond between the amide of Gly541' and the carbonyl of Gly534' in the β -turn- β motif could help to stabilize the C-terminal disulfide bond in a strained conformation. This would also explain why deletion of Gly541' results in a large loss of Trx reductase activity.

In contrast to the nucleophile-electrophile rationales presented above, an alternative explanation for the low Trx reductase activity of the mutants containing hCys could be the disruption of the hydrogen bonding network between the His438'-water dyad and Cys535', an interaction proposed by Becker and colleagues to aid in deprotonating the resolving Cys residue for the subsequent resolution of the mixed disulfide between PfTR and PfTrx in the "out" conformation.⁷ Substituting Cys535' with hCys might disrupt optimal hydrogen bond distances and result in low activity. However, the low Trx reductase activity of enzyme 10 cannot be due to a disruption of the interaction between Cys535' and the His438'-water dyad, as this interaction is preserved in this mutant. Our proposed electrophilic activation mechanism may explain the low activity observed in enzyme 10. The fact that mutation of His438' to Ala results in a 50% decrease in V_{\max} ⁷ while the replacement of Cys535' or Cys540' with hCys results in a more severe (>95%) activity is consistent with our electrophilic activation model. We caution the reader not to confuse His438' with His509'. As noted by Becker and co-workers, His438' is unique to PfTR and absent in other high- M_r TRs.⁷

CONCLUSIONS

We have investigated the unique C-terminal redox center of PfTR through mutagenesis and semisynthesis. Our results demonstrate the importance of a hydrogen bond between the amide NH group of Gly541' and the carbonyl of Gly534' in the β -turn- β motif to catalysis and the importance of disulfide ring size in the catalytic mechanism. We hypothesize that PfTR uses an electrophilic activation mechanism to help make the C-terminal disulfide reactive for thiol-disulfide exchange, but more work needs to be done to test our proposal. The results for the Sec for Cys substitution experiment suggest that PfTR evolved a unique C-terminal redox center, distinct from that found in humans and *A. gambiae*, so that PfTR could preferentially recognize its cognate substrate, PfTrx, relative to human Trx and *A. gambiae* Trx.

ASSOCIATED CONTENT

Supporting Information

Concentrations of PfTR constructs in the Trx reductase activity assays (Table S1), PfTR enzyme concentrations in the selenocysteine reductase assays (Table S2), gels of the fractions of the purification process from PfTR-thrombin constructs analyzed by SDS-PAGE (Figure S1), and Michaelis-Menten plots for enzymes 1, 12, and 13 (Figure S2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS

A_{340} , absorbance at 340 nm; Ala, alanine; β ME, β -mercaptoethanol; Cys, cysteine; Cys_{IC}, interchange cysteine; Cys_{CT}, charge-transfer cysteine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DmTR, *D. melanogaster* thioredoxin reductase; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; Fmoc, fluorenylmethyloxycarbonyl; Glu, glutamic acid; Gly, glycine; GST, glutathione S-transferase; His, histidine; H₂O₂, hydrogen peroxide; IPTG, isopropyl β -D-1-thiogalactopyranoside; Lys, lysine; MOPS, 3-(N-morpholino)propanesulfonic acid; NMA, N-(methyl)mercaptoacetamide; M_r , molecular ratio; NADPH, reduced β -nicotinamide adenine dinucleotide phosphate; PfTR, *P. falciparum* thioredoxin reductase; Sec, selenocysteine; Ser, serine; Trx, thioredoxin; TR, thioredoxin reductase; U, one-letter code for Sec; WT, wild type.

ADDITIONAL NOTES

^aWe use subscripted numbers 1 and 2 to designate each of the two Cys residues of the C-terminal redox center. The N-terminal Cys residue is designated as Cys₁, while the C-terminal Cys residue is designated as Cys₂.

^bWe refer to the N-terminal redox center by the amino acid sequence C_{CT}VNVGC_{IC}, where Cys_{CT} denotes the Cys residue that forms a thiolate charge-transfer complex with the flavin and Cys_{IC} denotes the interchange Cys thiolate that attacks the disulfide bond of the C-terminal redox center as is depicted in Figure 2.

^cThe prime designation denotes amino acid residues on the opposite subunit of the PfTR homodimer.

REFERENCES

- (1) Mustacich, D., and Powis, G. (2000) Thioredoxin reductase. *Biochem. J.* 346 (Part 1), 1–8.
- (2) Zhong, L., Arnér, E. S., Ljung, J., Aslund, F., and Holmgren, A. (1998) Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. *J. Biol. Chem.* 273, 8581–8591.
- (3) Williams, C. H., Arscott, L. D., Muller, S., Lennon, B. W., Ludwig, M. L., Wang, P. F., Veine, D. M., Becker, K., and Schirmer, R. H. (2000) Thioredoxin reductase two modes of catalysis have evolved. *Eur. J. Biochem.* 267, 6110–6117.
- (4) Bauer, H., Massey, V., Arscott, L. D., Schirmer, R. H., Ballou, D. P., and Williams, C. H., Jr. (2003) The mechanism of high M_r thioredoxin reductase from *Drosophila melanogaster*. *J. Biol. Chem.* 278, 33020–33028.
- (5) Lennon, B. W., Williams, C. H., Jr., and Ludwig, M. L. (2000) Twists in catalysis: Alternating conformations of *Escherichia coli* thioredoxin reductase. *Science* 289, 1190–1194.
- (6) Lothrop, A. P., Snider, G. W., and Hondal, R. J. (2014) Why is mammalian TR1 so dependent upon the use of selenium? *Biochemistry*, DOI: 10.1021/bi400651x.
- (7) Fritz-Wolf, K., Jortzik, E., Stumpf, M., Preuss, J., Iozef, R., Rahlfs, S., and Becker, K. (2013) Crystal structure of the *Plasmodium falciparum*

thioredoxin reductase-thioredoxin complex. *J. Mol. Biol.* 425, 3446–3460.

(8) Muller, S., Becker, K., Bergmann, B., Schirmer, R. H., and Walter, R. D. (1995) *Plasmodium falciparum* glutathione reductase exhibits sequence similarities with the human host enzyme in the core structure but differs at the ligand-binding sites. *Mol. Biochem. Parasitol.* 74, 11–18.

(9) Gilberger, T. W., Bergmann, B., Walter, R. D., and Muller, S. (1998) The role of the C-terminus for catalysis of the large thioredoxin reductase from *Plasmodium falciparum*. *FEBS Lett.* 425, 407–410.

(10) Gladyshev, V. N., Krause, M., Xu, X. M., Korotkov, K. V., Kryukov, G. V., Sun, Q. A., Lee, B. J., Wootton, J. C., and Hatfield, D. L. (1999) Selenocysteine-containing thioredoxin reductase in *C. elegans*. *Biochem. Biophys. Res. Commun.* 259, 244–249.

(11) Krnajsiki, Z., Gilberger, T. W., Walter, R. D., Cowman, A. F., and Muller, S. (2002) Thioredoxin reductase is essential for the survival of *Plasmodium falciparum* erythrocytic stages. *J. Biol. Chem.* 277, 25970–25975.

(12) Boumis, G., Giardina, G., Angelucci, F., Bellelli, A., Brunori, M., Dimastrogiovanni, D., Saccoccia, F., and Miele, A. E. (2012) Crystal structure of *Plasmodium falciparum* thioredoxin reductase, a validated drug target. *Biochem. Biophys. Res. Commun.* 425, 806–811.

(13) Banerjee, A. K., Arora, N., and Murty, U. S. (2009) Structural model of the *Plasmodium falciparum* thioredoxin reductase: A novel target for antimalarial drugs. *J. Vector Borne Dis.* 46, 171–183.

(14) Jortzik, E., and Becker, K. (2012) Thioredoxin and glutathione systems in *Plasmodium falciparum*. *Int. J. Med. Microbiol.* 302, 187–194.

(15) Andricopulo, A. D., Akoachere, M. B., Krogh, R., Nickel, C., McLeish, M. J., Kenyon, G. L., Arscott, L. D., Williams, C. H., Jr., Davioud-Charvet, E., and Becker, K. (2006) Specific inhibitors of *Plasmodium falciparum* thioredoxin reductase as potential antimalarial agents. *Bioorg. Med. Chem. Lett.* 16, 2283–2292.

(16) Krnajsiki, Z., Gilberger, T. W., Walter, R. D., and Muller, S. (2001) The malaria parasite *Plasmodium falciparum* possesses a functional thioredoxin system. *Mol. Biochem. Parasitol.* 112, 219–228.

(17) McMillan, P. J., Arscott, L. D., Ballou, D. P., Becker, K., Williams, C. H., Jr., and Muller, S. (2006) Identification of acid-base catalytic residues of high- M_r thioredoxin reductase from *Plasmodium falciparum*. *J. Biol. Chem.* 281, 32967–32977.

(18) Fritz-Wolf, K., Kehr, S., Stumpf, M., Rahlfs, S., and Becker, K. (2011) Crystal structure of the human thioredoxin reductase-thioredoxin complex. *Nat. Commun.* 2, 383.

(19) Gromer, S., Johansson, L., Bauer, H., Arscott, L. D., Rauch, S., Ballou, D. P., Williams, C. H., Jr., Schirmer, R. H., and Arnér, E. S. (2003) Active sites of thioredoxin reductases: Why selenoproteins? *Proc. Natl. Acad. Sci. U.S.A.* 100, 12618–12623.

(20) Bauer, H., Gromer, S., Urbani, A., Schnolzer, M., Schirmer, R. H., and Muller, H. M. (2003) Thioredoxin reductase from the malaria mosquito *Anopheles gambiae*. *Eur. J. Biochem.* 270, 4272–4281.

(21) Kanzok, S. M., Fechner, A., Bauer, H., Ulschmid, J. K., Muller, H. M., Botella-Munoz, J., Schneuwly, S., Schirmer, R., and Becker, K. (2001) Substitution of the thioredoxin system for glutathione reductase in *Drosophila melanogaster*. *Science* 291, 643–646.

(22) Evans, T. C., Jr., Benner, J., and Xu, M.-Q. (1998) Semisynthesis of cytotoxic proteins using a modified protein splicing element. *Protein Sci.* 7, 2256–2264.

(23) Eckenroth, B., Harris, K., Turanov, A. A., Gladyshev, V. N., Raines, R. T., and Hondal, R. J. (2006) Semisynthesis and characterization of mammalian thioredoxin reductase. *Biochemistry* 45, 5158–5170.

(24) Eckenroth, B. E., Lacey, B. M., Lothrop, A. P., Harris, K. M., and Hondal, R. J. (2007) Investigation of the C-terminal redox center of high- M_r thioredoxin reductase by protein engineering and semisynthesis. *Biochemistry* 46, 9472–9483.

(25) Harris, K. M., Flemer, S., Jr., and Hondal, R. J. (2007) Studies on deprotection of cysteine and selenocysteine side chain protecting groups. *J. Pept. Sci.* 13, 81–93.

(26) Flemer, S., Jr., Lacey, B. M., and Hondal, R. J. (2008) Synthesis of peptide substrates for mammalian thioredoxin reductase. *J. Pept. Sci.* 14, 637–647.

(27) Arnér, E. S., Zhong, L., and Holmgren, A. (1999) Preparation and assay of mammalian thioredoxin and thioredoxin reductase. *Methods Enzymol.* 300, 226–239.

(28) Lothrop, A. P., Ruggles, E. L., and Hondal, R. J. (2009) No selenium required: Reactions catalyzed by mammalian thioredoxin reductase that are independent of a selenocysteine residue. *Biochemistry* 48, 6213–6223.

(29) Björnstedt, M., Hamberg, M., Kumar, S., Xue, J., and Holmgren, A. (1995) Human thioredoxin reductase directly reduces lipid hydroperoxides by NADPH and selenocystine strongly stimulates the reaction via catalytically generated selenols. *J. Biol. Chem.* 270, 11761–11764.

(30) Sekharam, M., Cunnick, J. M., and Wu, J. (2000) Involvement of lipoxygenase in lysophosphatidic acid-stimulated hydrogen peroxide release in human HaCaT keratinocytes. *Biochem. J.* 346 (Part 3), 751–758.

(31) Sandalova, T., Zhong, L., Lindqvist, Y., Holmgren, A., and Schneider, G. (2001) Three-dimensional structure of a mammalian thioredoxin reductase: Implications for mechanism and evolution of a selenocysteine-dependent enzyme. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9533–9538.

(32) Carugo, O., Cemazar, M., Zahariev, S., Hudáky, I., Gáspári, Z., Perczel, A., and Pongor, S. (2003) Vicinal disulfide turns. *Protein Eng.* 16, 637–639.

(33) Gromer, S., Arscott, L. D., Williams, C. H., Jr., Schirmer, R. H., and Becker, K. (1998) Human placenta thioredoxin reductase: Isolation of the selenoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds. *J. Biol. Chem.* 273, 20096–20101.

(34) Lacey, B. M., and Hondal, R. J. (2006) Characterization of mitochondrial thioredoxin reductase from *C. elegans*. *Biochem. Biophys. Res. Commun.* 346, 629–636.

(35) Hazebrouck, S., Camoin, L., Faltin, Z., Strosberg, A. D., and Eshdat, Y. (2000) Substituting selenocysteine for catalytic cysteine 41 enhances enzymatic activity of plant phospholipid hydroperoxide glutathione peroxidase expressed in *Escherichia coli*. *J. Biol. Chem.* 275, 28715–28721.

(36) Lobanov, A. V., Delgado, C., Rahlfs, S., Novoselov, S. V., Kryukov, G. V., Gromer, S., Hatfield, D. L., Becker, K., and Gladyshev, V. N. (2006) The *Plasmodium* selenoproteome. *Nucleic Acids Res.* 34, 496–505.

(37) Roseler, A., Prieto, J. H., Iozef, R., Hecker, B., Schirmer, R. H., Kulzer, S., Przyborski, J., Rahlfs, S., and Becker, K. (2012) Insight into the selenoproteome of the malaria parasite *Plasmodium falciparum*. *Antioxid. Redox Signaling* 17, 534–543.

(38) Arnér, E. S. (2010) Selenoproteins: What unique properties can arise with selenocysteine in place of cysteine? *Exp. Cell Res.* 316, 1296–1303.

(39) Hondal, R. J., and Ruggles, E. L. (2011) Differing views of the role of selenium in thioredoxin reductase. *Amino Acids* 41, 73–89.

(40) Lothrop, A. P., Snider, G. W., Flemer, S., Jr., Ruggles, E. L., Davidson, R. S., Lamb, A., and Hondal, R. J. (2013) Compensating for the absence of selenocysteine in high molecular weight thioredoxin reductases: The electrophilic activation hypothesis. *Biochemistry*, accepted.