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Why Is Mammalian Thioredoxin Reductase 1 So Dependent upon the Use of Selenium?

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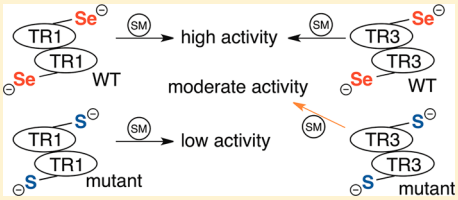
Supporting Information

ABSTRACT: Cytosolic thioredoxin reductase 1 (TR1) is the best characterized of the class of high-molecular weight (M_r) thioredoxin reductases (TRs). TR1 is highly dependent upon the rare amino acid selenocysteine (Sec) for the reduction of thioredoxin (Trx) and a host of small molecule substrates, as mutation of Sec to cysteine (Cys) results in a large decrease in catalytic activity for all substrate types. Previous work in our lab and others has shown that the mitochondrial TR (TR3) is much less dependent upon the use of Sec for the reduction of small molecules. The Sec-dependent substrate utilization behavior of TR1 may be the exception and not the rule as we show that a variety of high-M_r TRs from other organisms, including *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Plasmodium falciparum*, do not require Sec to reduce small molecule substrates, including 5,5′-dithiobis(2-nitrobenzoic acid), lipoic acid, selenite, and selenocystine. The data show that high-M_r TRs can be divided into two groups based upon substrate utilization patterns: a TR1 group and a TR3-like group. We have constructed mutants of TR3-like enzymes from mouse, *D. melanogaster*, *C. elegans*, and *P. falciparum*, and the kinetic data from these mutants show that these enzymes are less dependent upon the use of Sec for the reduction of substrates. We posit that the mechanistic differences between TR1 and the TR3-like enzymes in this study are due to the presence of a "guiding bar", amino acids 407−422, found in TR1, but not TR3-like enzymes. The guiding bar, proposed by Becker and co-workers [Fritz-Wolf, K., Urig, S., and Becker, K. (2007) The structure of human thioredoxin reductase 1 provides insights into C-terminal rearrangements during catalysis. *J. Mol. Biol. 370*, 116−127], restricts the motion of the C-terminal tail containing the C-terminal Gly-Cys-Sec-Gly, redox active tetrapeptide so that only this C-terminal redox center can be reduced by the N-terminal redox center, with the exclusion of most other substrates. This makes TR1 highly dependent upon the use of Sec because the selenium atom is responsible for both accepting electrons from the N-terminal redox center and donating them to the substrate in this model. Loss of both *Se*-electrophilicity and *Se*-nucleophilicity in the Sec → Cys mutant of TR1 greatly reduces catalytic activity. TR3-like enzymes, in contrast, are less dependent upon the use of Sec because the absence of the guiding bar in these enzymes allows for greater access of the substrate to the N-terminal redox center and because they can make use of alternative mechanistic pathways that are not available to TR1.



Thioredoxin reductase (TR) is a redox active, homodimeric flavoenzyme that belongs to the pyridine nucleotide disulfide oxidoreductase family, whose members also include glutathione reductase (GR), mercuric ion reductase, and lipoamide dehydrogenase.[1] TRs exist in both a low-M_r (~35 kDa subunits) form found in both prokaryotes and lower eukaryotes and a high-M_r (~55 kDa subunits) form found in higher eukaryotes.[1,2] These two classes employ different catalytic strategies to perform the same overall reaction because of large differences in structure. While both classes of TRs utilize NADPH as the ultimate source of electrons in the reduction of the small protein thioredoxin (Trx), the two classes of enzymes differ in the final transfer mechanism of these electrons to Trx. The low-M_r TRs have two redox centers (a noncovalently associated FAD and an N-terminal dithiol/disulfide pair) and must undergo a large conformational change to reduce their target substrate.[3,4] In contrast, high-M_r TRs contain FAD, a N-terminal dithiol/disulfide pair, and a third, C-terminal redox center that delivers electrons to Trx.[2]

High-M_r TRs can be further classified as type I or type II based on their respective C-terminal redox motifs. Type I TRs utilize a vicinal disulfide bond with an X-Cys_1-Cys_2-X sequence (we designate the N-terminal Cys residue of the dyad as residue "1" and the C-terminal Cys residue of the dyad as residue "2").[5−7] Vicinal disulfides are an uncommon motif in proteins, with the resulting eight-membered ring structure found relatively few times in protein structures.[8] This type of TR can be even further differentiated into two subtypes depending on the usage of the rare amino acid selenocysteine (Sec or U) in the penultimate position in place of Cys_2.[9,10] We denote type Ia as those TRs that use Sec and type Ib as those that use Cys. A second, distinct type of high-M_r TRs is used by apicomplexan protists such as *Plasmodium falciparum*, *Toxoplasma gondii*, and *Cryptosporidium parvum*, which we denote as type II.[11] The C-

Received: May 23, 2013
Revised: January 4, 2014
Published: January 6, 2014

Table 1. Comparison of TR1 and TR3 Wild-Type and Mutant Enzyme Activities toward Various Substrates[a]

Enzyme		Activity [mol of NADPH min^{-1} (mol of TR)$^{-1}$]				
		Trx[b] (5 μM)	DTNB (5 mM)	Lipoic acid (1 mM)	Selenite (0.1 mM)	Se-cystine (50 μM)[c]
type Ia TR1						
cl TR1-G*C*U*C*G[d]	**1**	1667	1333	297	471	102
cl TR1-GCCG[d]	**2**	18	63	0.8	43	2.1
cl TR1-GCSG[d]	**3**	NA[e]	17	0.9	3.5	1.1
type Ia TR3						
mTR3-G*C*U*C*G	**4**	162 ± 6	1020 ± 30	11.5 ± 1.0	110 ± 3.0	235 ± 35
mTR3-GCCG	**5**	0.5 ± 0.1	605 ± 20	5.2 ± 1.4	41 ± 2	44 ± 4
mTR3-GCSG	**6**	NA[e]	400 ± 15	1.8 ± 0.3	14.6 ± 0.2	1.0 ± 0.4
mTR3-GSCG	**7**	NA[e]	700 ± 150	7.4 ± 0.3	22 ± 1	24 ± 2
mTR3-GSSG	**8**	NA[e]	990 ± 290	4.5 ± 0.4	15.0 ± 5.5	0.5 ± 0.1
type Ib TRs						
DmTR-S*C*C*S*	**9**	13.1 ± 1.4	146 ± 6	2.4 ± 0.9	17.3 ± 1.4	45.5 ± 13.3
CeTR-G*C*C*G*	**10**	21 ± 3[f]	130 ± 26	1.9 ± 0.3	23.4 ± 1.0	50 ± 2
type II TR						
PfTR-G*C*GGGK*C*G	**11**	37 ± 7	395 ± 5	24 ± 1	53 ± 4	170 ± 23
PfTR-GSGGGKCG	**12**	NA[e]	384 ± 8	27 ± 1	27 ± 4	11 ± 2
PfTR-GCGGGK*S*G	**13**	NA[e]	383 ± 19	32 ± 1	32 ± 3	41 ± 5
PfTR-GSGGGK*S*G	**14**	NA[e]	437 ± 1	35 ± 1	21 ± 3	0.6 ± 0.4

[a]The enzymes in this study are abbreviated with the name TR representing the body of the enzyme followed by the amino acid sequence of the C-terminal redox center. A prefix is added to denote the TRs from different species. The abbreviations are as follows: clTR1-GCUG, calf liver cytosolic TR; mTR3, mouse mitochondrial TR; DmTR, *D. melanogaster* TR; CeTR, *C. elegans* mitochondrial TR; PfTR, *P. falciparum* TR. [b]TR1 studies utilized human Trx, while we utilized *E. coli* Trx for the TR3-like enzymes. [c]TR1 values are for 45 μM selenocystine. [d]Values taken from ref 9. [e]No activity. [f]At 90 μM Trx. We could not establish an activity for Trx concentrations of <90 μM.

terminal redox center of these type II TRs has an amino acid composition much different compared to that of type I TRs. For example, the type II TR from *P. falciparum* has a C-terminal redox center with a Gly-Cys$_1$-Gly-Gly-Gly-Lys-Cys$_2$-Gly sequence.[11−13]

Humans as well as other higher eukaryotes have three genes for type Ia thioredoxin reductase: a cytosolic enzyme (TrxR1 or TR1), a mitochondrial form (TrxR2 or TR3), and a testis specific thioredoxin/glutathione reductase (TGR).[14−16] TGR is a multifunctional enzyme that can reduce glutathione disulfide and the disulfide bond of Trx.[14,17] In this report, the cytosolic TR will be termed TR1 and the mitochondrial enzyme TR3. Each of these three genes contains an in-frame UGA codon that encodes the Sec residue. While mutation of Sec to Cys in each of these three enzymes results in a large decrease in catalytic activity,[9,18] Sec is not catalytically essential to the reduction of Trx because of the presence of type Ib and type II Cys orthologs that catalyze identical reactions with comparable efficiencies.[19,20] The chemical and biological function of Sec in TR and other Sec-containing enzymes is the subject of ongoing debate in the field.[19,21,22]

Of the three type Ia TRs of higher eukaryotes, TR1 has been studied for the longest period of time and the most well characterized. TR1 has been found to reduce a broad range of macromolecular and small molecule substrates (discussed in ref 9). The broad substrate specificity of TR1 has been attributed to the presence of the rare Sec residue.[20] Deletion of Sec or mutation of Sec to Cys in TR1 results in a mutant enzyme whose catalytic activity toward many small molecule substrates such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), lipoic acid, selenite, and selenocystine is greatly impaired.[9]

In contrast to the work on TR1, studies in our laboratory indicate that the mouse mitochondrial TR (mTR3) behaves quite differently with respect to the dependence on Sec for reduction of small molecule substrates.[20,23] While the human mitochondrial TR has not been as extensively characterized, a study by Rackham and co-workers showed that it could reduce DTNB and lipoamide at pH 8.0 without the use of Sec.[24] Similarly, TGR from *Schistosoma mansoni* does not need Sec to reduce some small molecule substrates.[25] We have also shown that Cys-containing type Ib TRs do not require Sec to reduce certain small molecule substrates.[7,18] The data summarized here show that Sec is needed to impart broad substrate specificity to TR1 only.

In this study, we have summarized all of the steady-state kinetic data with various small molecule substrates for type Ia and type Ib TRs to demonstrate that TR1 is the exception, not the rule, with respect to the dependence on Sec for the reduction of small molecule substrates. To bolster our case, we show here that the type II TR from *P. falciparum* (PfTR) is dissimilar from TR1 and exhibits behavior identical to that of the mitochondrial TR and type Ib Cys-TRs with respect to substrate utilization. We have constructed various mutants in the C-terminal redox center of mouse TR3 and PfTR and show that these enzymes can utilize a catalytic pathway that is independent of the C-terminal redox center for the reduction of various small molecule substrates. Previous structural studies of human TR1 have raised the possibility that TR1 and TR3 may operate differently with respect to apparent differences in their flexible C-terminal tails that contain the third redox active center,[26,27] which may offer a potential explanation for the kinetic differences observed between TR1 and TR3 and multiple Cys-TR orthologs. The data presented here support this structural observation.

MATERIALS AND METHODS

Materials. NADPH and racemic (*R*,*S*)-lipoic acid were from Sigma-Aldrich (St. Louis, MO). Fmoc-L-Cys(Trt) and Fmoc-D-Cys(Trt) amino acids were from RS Synthesis (Louisville, KY) and NovaBiochem (San Diego, CA), respectively. Wang resin

was also from NovaBiochem. Glutathione Fast Flow resin and bovine thrombin were from GE Healthcare (Waukesha, WI). Plasmids pTYB1, pTYB3, T4 DNA ligase, and ER2566 *Escherichia coli* cells were from New England Biolabs (Ipswich, MA). DNA purification kits were from Qiagen. Production and purification of *E. coli* Trx have been previously described.[18] All other reagents were purchased from either Fisher Scientific (Fair Lawn, NJ) or Sigma-Aldrich and were of reagent grade or better.

Enzyme Expression and Purification. Expression of mouse mitochondrial TR (mTR3), *Drosophila melanogaster* TR (DmTR), and *Caenorhabditis elegans* mitochondrial TR (CeTR) and their truncated Δ8 mutants have been previously described.[7,18,28−30] Production of *P. falciparum* TR (PfTR) and PfTRΔ7 is described below.

mTR-GSSG Expression and Purification. The full-length mTR3 Cys mutant construct (mTR-GCCG[a]) DNA was used as a template to create a mutant in which both C-terminal redox active residues were mutated to Ser (enzyme **8** in Table 1). The sequences of the DNA primers used in polymerase chain reactions (PCRs) were 5′-AACAGACCATGGGAGGG-CAGCAGAGCTTT-3′ (upstream primer) and 5′-ACAGCC-GCTCTTCAGCAGCCGCTACTACCAGTCACAGTAGGC-TCCAGGCC-3′ (downstream primer). Amplified DNA and plasmid pTYB3 were each digested with restriction enzymes NcoI and SapI at 37 °C for 2 h and then purified with a Qiaprep Spin Miniprep Kit. The two DNA fragments were ligated with *E. coli* T4 DNA ligase, yielding a plasmid encoding mTR-GSSG as an intein−chitin binding domain fusion protein. Expression and purification of the mutant TR were identical to those of the wild-type (WT) enzyme and similar mutants as described previously.[20]

PfTR Expression and Purification. The expression plasmid encoding the full-length PfTR-CGGGKCG enzyme was kindly provided by F. Angellucci of the University of Rome (Rome, Italy). The PfTR gene was contained in a pGEX-4T-1 vector that encodes a GST fusion protein. The full-length PfTR WT enzyme, as a GST fusion construct, was expressed in 6 L of *E. coli* ER2566 cells containing 0.2 mg/mL ampicillin at 37 °C until the OD_{600} reached 0.6. The cells were cooled to 20 °C, and the expression was induced by the addition of IPTG to a final concentration of 0.5 mM. After overnight incubation at 20 °C, the cells were harvested by centrifugation (Beckman J2-21 centrifuge, JA-14 rotor, 10000 rpm). The cells were homogenized in buffer A [50 mM Tris, 200 mM NaCl, and 2 mM βME (pH 7.4)] with the inclusion of 1 mg/mL chicken egg lysozyme to help degrade bacterial cell walls. The cells were further lysed by probe sonication, and the solution was cleared by centrifugation at 12000 rpm for 1 h at 4 °C.

Cleared lysate was gravity-loaded onto glutathione Sepharose 4 fast flow resin pre-equilibrated with buffer A. The bound PfTR was washed extensively with buffer A, and the resin slurry was transferred to two 50 mL conical vials. Bovine thrombin (20 units) supplemented with 5 mM $CaCl_2$ was added to the resin/protein slurry overnight at 4 °C while it was being mixed to induce cleavage of the GST fusion protein to produce free PfTR.

PfTRΔ7 Purification. The full-length PfTR construct in pGEX-4T-1 was utilized as a template to generate a truncated mutant that lacks the final seven C-terminal amino acids (PfTRΔ7). This was accomplished by amplifying the DNA via PCR with the upstream primer 5′-AACAGACATATGGGAT-CCTGCAAA-3′ and the downstream primer 5′-ACAGCCG-CTCTTCAGCAACCACCTTT-3′, which introduced NdeI and SapI restriction sites, respectively. The amplified PfTRΔ7 DNA and plasmid pTYB1 were digested with NdeI and SapI at 37 °C for 2 h, purified with a Qiaprep Spin Miniprep Kit, and subsequently ligated with *E. coli* T4 DNA ligase. The pTYB1 plasmid encodes an intein−chitin binding domain fusion protein and allows for the liberation of TR from the intein via cleavage by a small molecule thiol.[18] Verification of the PfTRΔ7 mutant plasmid was performed at the University of Vermont DNA sequencing facility. Truncated PfTRΔ7 and the full-length enzyme were expressed identically. Purification of the PfTRΔ7−intein−CBD fusion protein was the same as described for mammalian TR constructs.[20,31] Briefly, PfTRΔ7 cleaved from the chitin resin was concentrated and exchanged into buffer B [50 mM potassium phosphate and 500 mM NaCl (pH 8.0)]. For the final purification step, the truncated enzyme was loaded onto a S200-Sephacryl gel filtration column pre-equilibrated with buffer B. The purity of PfTRΔ7 was judged by SDS−PAGE analysis.

Peptide Synthesis. Trimer peptides with D-Cys-L-Cys-Ser and L-Cys-D-Cys-Ser sequences were synthesized on Wang resin using standard Fmoc chemistry. Peptides were cleaved from the resin using a mixture of 95% trifluoroacetic acid, 2.5% water, and 2.5% ethanedithiol. After precipitation in cold diethyl ether, the dried peptides were dissolved in water with 5−10% acetonitrile, lyophilized, and analyzed by both HPLC and MALDI-TOF mass spectrometry.

Production of Semisynthetic Enzymes. For the production and purification of semisynthetic DmTR enzymes, we followed our previously published protocol.[18,31] Purity was judged by SDS−PAGE analysis, and concentrations of homodimeric enzymes were determined spectrophotometrically, using a 22.6 mM^{-1} cm^{-1} extinction coefficient for FAD.

Kinetic Assays. All TR enzymes were assayed with a variety of different substrates. Reductions of Trx, DTNB, selenocystine, lipoic acid, and selenite have been previously described.[9,20] Briefly, assays contained potassium phosphate buffer (50 mM for Trx reductase assays, 500 mM for selenocystine reductase assays, and 100 mM for all other substrates), 1 mM EDTA, 150 μM NADPH, and varying concentrations of each substrate. Enzyme concentrations utilized in each assay can be found in Tables S1 and S2 of the Supporting Information. The change in absorbance at 412 nm was used for DTNB to monitor production of the thionitrobenzoate anion, and the change in absorbance at 340 nm was followed to monitor the consumption of NADPH for all other assays. For steady-state assays to measure the DTNB reductase activity of various TRs, the concentration of DTNB was varied from 0.1 to 4 mM while the concentration of NADPH was held constant at 150 μM. Plots of initial velocity versus substrate concentration were constructed, and the curves were fit to the Michaelis−Menten equation to determine k_{cat} and K_m.

Sequence Alignment of TR1 and TR3-like Enzymes. The primary amino acid sequences for TR1 enzymes (*Homo sapiens* and *Rattus norvegicus*) and TR3-like enzymes (*H. sapiens*, *Mus musculus*, *D. melanogaster*, *C. elegans*, and *P. falciparum*) discussed in this study were aligned using ClustalW2.[32] Pairwise alignment was conducted using the default program settings. The alignment was used to examine the conservation of guiding bar residues among the various TRs.

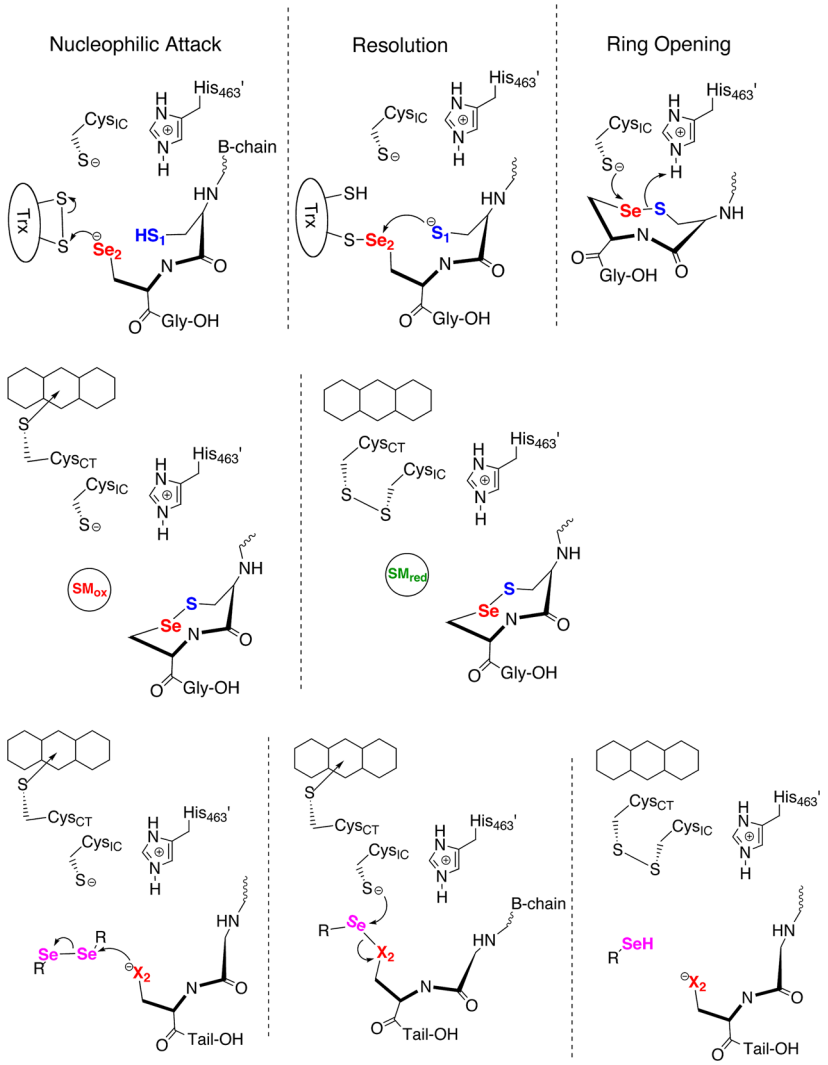


Figure 1. Three different proposed substrate utilization pathways in high-M_r TRs. In path A (top row), TR1 is physically constrained to use this pathway for the reduction of Trx and other small molecule substrates in this study. For the sake of brevity, we show only part of the catalytic mechanism. For a complete description of all of the steps in the catalytic mechanism of high-M_r TRs, please see ref 6. The active site of type Ia TRs consists of a vicinal Cys-Sec dyad, a N-terminal disulfide/dithiol pair consisting of Cys_{IC} (interchange Cys) and Cys_{CT} (change-transfer Cys), and a His residue that functions as an acid−base catalyst. PfTR has a similar arrangement of residues with the exception of a C-terminal redox center with a CGGGKC sequence that forms a 20-membered disulfide ring upon oxidation. There are three basic chemical steps in path A: (i) nucleophilic attack of the selenolate on the substrate disulfide bond (Trx or another small molecule), (ii) resolution of the mixed selenosulfide bond between the enzyme and substrate to form the oxidized, eight-membered ring of the C-terminal tail, and (iii) attack of the thiolate of Cys_{IC} on the Se atom of the dyad. Type Ib TRs should have an identical mechanism but use Cys in place of Sec (from left to right, respectively). Type II TRs should have a similar mechanism, with the difference being the use of a 20-membered C-terminal disulfide ring. In path B (middle row), TR3-like enzymes can use the N-terminal redox center to reduce small molecules (SM) like DTNB, lipoic acid, and selenite. In this mechanism, Cys_{IC} would initially attack the small molecule substrate to form a covalently bound intermediate. This intermediate would be resolved by attack of the thiolate of Cys_{CT} on the sulfide of Cys_{IC}, resulting in an oxidized (disulfide) N-terminal redox center. Path C (bottom row) is the proposed mechanism for the reduction of selenocystine by mutant TR3-like enzymes. Here X_2 = S or Se. The data show that variants of TR3-like enzymes need to contain only a single sulfhydryl group to reduce selenocystine because the S−Se_{sub} bond that is formed after the initial attack step is capable of being reduced by the N-terminal redox center as shown. The data show that path A is probably the favored pathway for reduction of selenocystine by the WT enzymes.

RESULTS AND DISCUSSION

Summary of Substrate Utilization by Different TR Types. A seminal study by Zhong and Holmgren of calf liver TR1 (a type Ia TR) showed that Sec was required to reduce both macromolecular and small molecule substrates.[9] The results of this earlier study are listed in Table 1 along with the same data as determined in this study for the type Ia mouse mitochondrial enzyme, type Ib enzymes from *D. melanogaster* and *C. elegans*, and the type II TR from *P. falciparum*.

The Data Indicate Three Mechanistic Pathways Are Possible. We believe our collective data indicate that there are three mechanistic pathways for substrate utilization in high-M_r TRs. These are shown in Figure 1 and are (i) path A, which requires the full participation of both N- and C-terminal redox centers for the reduction of all types of substrates listed in Table 1, (ii) path B, where substrates such as DTNB, lipoic acid, and selenite can be reduced solely by the N-terminal redox center, and (iii) path C, which requires the N-terminal redox center and only a partially functional C-terminal redox center.

dx.doi.org/10.1021/bi400651x | *Biochemistry* 2014, 53, 554−565

Table 2. Comparison of the Activities of Truncated TRs with Various Substrates[a]

Enzyme		Activity [mol of NADPH min^{-1} (mol of TR)$^{-1}$]				
		Trx[b] (5 μM)	DTNB (5 mM)	Lipoic acid (1 mM)	Selenite (0.1 mM)	Se-cystine (50 μM)[c]
type Ia TR1						
cl TR1Δ2[d]	**15**	NA[e]	4.1	0.3	1.2	1.6
type Ia TR3						
mTR3Δ2	**16**	NA[e]	815 ± 42	6.5 ± 0.4	23 ± 2	8.2 ± 1.4
mTR3Δ8	**17**	NA[e]	2430 ± 56	20.0 ± 0.2	18 ± 1	0.8 ± 0.4
type Ib TRs						
DmTRΔ8	**18**	NA[e]	520 ± 70	4.7 ± 0.7	12.4 ± 1.9	3.3 ± 2.2
CeTRΔ8	**19**	NA[e]	130 ± 50	4.2 ± 0.1	16 ± 1	2.2 ± 0.8
type II TR						
PfTRΔ7	**20**	NA[e]	280 ± 7	32 ± 1	28 ± 2	0.6 ± 0.2

[a]The enzymes in this study are abbreviated with the name TR representing the body of the enzyme followed by the amino acid sequence of the C-terminal redox center. A prefix is added to denote the TRs from different species. The abbreviations are as follows: clTR1-GCUG, calf liver cytosolic TR; mTR3; mouse mitochondrial TR; DmTR, *D. melanogaster* TR; CeTR, *C. elegans* mitochondrial TR; PfTR. *P. falciparum* TR. [b]TR1 studies utilized human Trx, while we utilized *E. coli* Trx for the TR3-like enzymes. [c]TR1 values are for 45 μM selenocystine. [d]Values taken from ref 9. [e]No activity.

Below we discuss our evidence for dividing the TRs listed in Table 1 into two groups based upon the substrate utilization pathways shown in Figure 1. The first group is comprised solely of TR1, while the second group is comprised of TR3 and "TR3-like" enzymes, DmTR, CeTR, and PfTR. The data indicate that TR1 can mostly use only path A, whereas TR3-like enzymes can use all three pathways. Below we discuss each type of substrate separately and their dependence (or lack of) on the use of Sec. Some of the concepts we present here have been discussed previously by us for TR3, for example, the electrophilicity of the substrate.[20,33] However, the key concept that we are now proposing (supported by our data) is that there are mechanistic differences between TR1 and TR3-like enzymes, and this idea is new to the field.

Dependence of the Reduction of Macromolecular Trx on the Use of Sec. With respect to Sec-containing mammalian TRs (enzymes **1** and **4**), the data in Table 1 clearly demonstrate the necessity of Sec for the effective reduction of Trx by both TR1 and TR3 because mutation of Sec to Cys results in a very large decrease in activity (cf. **2** with **1** and **5** with **4** in Table 1). We do note the ∼10-fold greater Trx reductase activity of TR1 versus that of TR3 at a substrate concentration of 5 μM. This may be attributed to the use of human Trx in the TR1 assay,[9] while *E .coli* Trx was used to assay the activity with TR3. Enzymes **9**−**11** are Cys orthologs of the mammalian enzyme and are not dependent upon the use of Sec to reduce their respective cognate Trx substrates.[34] The activities reported here are with *E. coli* Trx, and these enzymes have much lower activity toward the noncognate, *E. coli* Trx substrate. The C-terminal redox center of mammalian TR contains a Cys-Sec redox dyad, and both residues are needed for catalysis of Trx. The adjacent Cys residue most likely is involved in resolving the mixed selenosulfide bond formed between TR and Trx, resulting in the formation of an eight-membered ring as shown path A in Figure 1. Mutation of the resolving Cys residue to Ser eliminates Trx reductase activity as can be seen by comparing the activities of enzymes **7** and **4** in Table 1. The Sec residue most likely functions as the residue involved in the initial nucleophilic attack on the disulfide bond of Trx.[27] Mutation of this Sec residue to Ser also results in a total loss of Trx reductase activity (compare **3** to **2** and **6** to **4** in Table 1). Possible reasons for the dependence on the use of Sec for the reduction of Trx will be discussed below. The requirement for a fully intact C-terminal redox center for the reduction of macromolecular Trx in all of the different types of TRs is demonstrated by a complete loss of activity when the C-terminal redox center is eliminated by truncation of the enzyme as demonstrated by the data in Table 2. While TR1 and TR3 are similar with respect to their requirement for the use of Sec in the reduction of their primary substrate Trx, mechanistic differences become readily apparent upon examination of the reduction of various types of small molecules as explained below.

Dependence of the Reduction of the Disulfide Bond of DTNB in TR1 but Not TR3-like Enzymes on the Use of Sec. TR1 retains only 5% of its ability to reduce DTNB when Sec is mutated to Cys (cf. enzymes **1** and **2** in Table 1). This is in marked contrast to the data for TR3, which retains 58% of its DTNB reductase activity when Sec is mutated to Cys (cf. enzymes **4** and **5**). A nearly identical result has been reported for human TR3 when the last two amino acids (Sec-Gly) are removed from the enzyme (hTR3Δ2), but the pH of the reaction must be increased to 8 to measure the enhanced activity.[24] Mutants in which the Cys-Sec dyad in TR3 has been altered to either a Cys-Ser dyad or a Ser-Cys dyad (enzymes **6** and **7**) also retain high DTNB reductase activity. To further examine the role of the C-terminal redox center in the mitochondrial enzyme, we created a "dead tail" mutant in which the Cys-Sec dyad was mutated to a Ser-Ser dyad (enzyme **8**). This full-length enzyme contains a C-terminus that is incapable of any redox chemistry, yet its DTNB reductase activity approaches that of the WT enzyme (enzyme **4**). These data indicate that the mitochondrial enzyme reduces the disulfide bond of DTNB using a mechanism that is independent of the C-terminal redox center, unlike the case for TR1. This is further supported by the data in Table 2, which show that a truncated mutant of TR3 (enzyme **17**) has even higher DTNB reductase activity than the WT enzyme, while a truncated TR1 enzyme has <1% of the WT activity remaining (cf. enzyme **15** with enzyme **1**).

We then constructed the analogous mutants with PfTR, a type II TR, to see whether the mutants would yield kinetic behavior more similar to that of TR1 or TR3 using DTNB as a substrate with respect to usage of the C-terminal redox center. The data in Table 1 indicate that PfTR mutant enzymes **12**−**14** utilize DTNB in a fashion analogous to that of TR3 mutant

Table 3. Comparison of the Steady-State Kinetic Parameters of TR1 and TR3-like Enzymes Utilizing DTNB as a Substrate[a]

Enzyme		k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})	k_{cat}/K_m(WT)/k_{cat}/K_m(mutant)
type Ia TR1					
rat TR1-G*C*U*C*G[b]	**1**	2040 ± 62	0.288 ± 0.023	7083	–
rat TR1-GCCG[b]	**2**	11.6 ± 0.1	0.044 ± 0.003	264	26.8
rat TR1Δ2[b]	**15**	<2	NA[c]	NA[c]	NA[c]
type Ia TR3					
mTR3-G*C*U*C*G	**4**	1250 ± 70	0.5 ± 0.1	2500	–
mTR3-GCCG	**5**	794 ± 78	1.8 ± 0.4	441	5.67
mTR3-GC**S**G	**6**	450 ± 70	2.2 ± 0.6	205	12.2
mTR3-G**S**CG	**7**	920 ± 1	2.9 ± 0.5	317	7.89
mTR3-G**S**S**S**G	**8**	1940 ± 120	5.4 ± 0.4	359	6.96
mTR3Δ2	**16**	1542 ± 302	5.0 ± 1.4	308	8.11
type Ib TRs					
DmTR-S*C*C*S*	**9**	100 ± 3	0.2 ± 0.02	500	–
DmTR-SC_DC_LS	**9A**	147 ± 7.6	0.4 ± 0.1	367.5	1.36
DmTR-SC_LC_DS	**9B**	200 ± 20	1.4 ± 0.4	142.8	3.5
CeTR-G*C*C*G*	**10**	134 ± 5	0.4 ± 0.1	335	–
type II TR					
PfTR-G*C*GGGK*C*G	**11**	436 ± 17	0.44 ± 0.06	991	–
PfTR-GSGGGK*C*G	**12**	477 ± 16	1.1 ± 0.1	433.6	2.28
PfTR-G*C*GGGK**S**G	**13**	477 ± 40	1.5 ± 0.3	318	3.11
PfTR-GSGGGK**S**G	**14**	478 ± 9	1.1 ± 0.1	434.5	2.28

[a]The enzymes in this study are abbreviated with the name TR representing the body of the enzyme followed by the amino acid sequence of the C-terminal redox center. A prefix is added to denote the TRs from different species. The abbreviations are as follows: clTR1-GCUG, calf liver cytosolic TR; mTR3, mouse mitochondrial TR; DmTR, *D. melanogaster* TR; CeTR, *C. elegans* mitochondrial TR; PfTR, *P. falciparum* TR. [b]Taken from ref 36. [c]Unable to determine.

enzymes **6**–**8**. This result is in total agreement with results reported by Gilberger and co-workers for analogous Cys → Ala substitutions.[35] These data indicate that PfTR must use a mechanism that is independent of the C-terminal redox center for the reduction of DTNB. This view is further supported by the data in Table 2 that show that the truncated PfTR (enzyme **20**) has DTNB reductase activity similar to that of the WT enzyme (enzyme **11**). While we did not construct the analogous serine mutants for the type Ib TRs, we previously constructed truncated enzymes missing the C-terminal redox center, and these enzymes (**18** and **19**) have DTNB reductase activity higher than that of or the same as that of their full-length WT counterparts.

While the data in Tables 1 and 2 compare the specific DTNB reductase activity of TR1 to those of the other TR3-like enzymes in this study, the data in Table 3 provide a direct comparison of steady-state catalytic parameters (k_{cat} and K_m). Just as the specific activity data show, analysis of the steady-state parameters in Table 3 shows TR1 is almost completely dependent upon the use of Sec for the reduction of DTNB, while all of the naturally occurring Cys-TRs as well as mTR3 can reduce DTNB in a Sec-independent fashion. Moreover, alteration of the C-terminal redox centers of these enzymes by mutation or deletion either increases the DTNB reductase activity (k_{cat}) or has a minimal effect. The data also show that the K_m for DTNB in these mutants increases moderately, in the range of 3–10-fold.

Further support for our idea that TR1 is distinct from TR3-like enzymes comes from comparison of the k_{cat}/K_m(WT)/k_{cat}/K_m(mutant) ratios in Table 3. For type Ib and type II TRs, this ratio is small, in the range of 1–3, while for TR1, this ratio is significantly larger (26.8). For the mutants of type Ia TR, this ratio is between (5–12) that of TR1 and that of the type Ib and type II TRs.

Use of Sec for the Reduction of Lipoic Acid and Selenite in TR1 and TR3-like Enzymes. The reduction of selenite by TR3, as well as type Ib and type II TRs in Table 1, is similar to that by TR1 but with some important differences that we note below. TR1 has more overall activity than any of the other enzymes in Table 1 toward selenite and therefore loses a greater percentage of its overall activity when Sec is mutated to Cys (enzymes **1** and **2**) compared to the same mutation in TR3 (enzymes **4** and **5**). In fact, enzyme **5** has activity toward selenite nearly identical to that of enzyme **2**.

A key difference between TR1 and the TR3-like enzymes listed in Table 1 is the ability of the N-terminal redox center in the latter group to reduce selenite in the absence of the C-terminal redox center, as the data in Table 2 makes apparent. For example, compare the activities of enzyme **16** or **17** with enzyme **15**. This comparison demonstrates that the N-terminal redox center of TR3 has an ∼15-fold stronger ability to reduce selenite than the same redox center of TR1. The same is true for the N-terminal redox centers of type Ib TRs; all of the catalytic power to reduce selenite appears to come from the N-terminal redox center in these enzymes [compare enzymes **9** and **10** with enzymes **18** and **19**, respectively (Tables 1 and 2)], even though the overall activities of the full-length enzymes are ∼20-fold lower than that of TR1. The type II TR also has lower overall activity toward selenite as a substrate than TR1 (compare enzymes **11** and **1** in Table 1), but approximately half of this activity comes from the N-terminal redox center in the type II TR (cf. enzyme **11** with enzymes **12**–**14**). This contrasts with TR1 in which the N-terminal redox center appears to contribute ∼1% of the selenite reductase activity [compare enzyme **15** with enzyme **1** (Tables 1 and 2)].

A somewhat different trend is observed when lipoic acid is used as the substrate. An analysis similar to the one presented above reveals that the N-terminal redox center of TR3 has an

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~25-fold stronger ability to reduce lipoic acid than the N-terminal redox center of TR1 (cf. enzyme **1** with enzyme **4**). While type 1b TRs have significantly lower lipoic acid reductase activity than TR1, a fair conclusion is that all this activity is due to the N-terminal redox center. An identical conclusion can be reached for the type II TR.

Some conclusions from the data in Tables 1−3 are as follows. DmTR, CeTR, and PfTR do not contain a Sec residue, but their patterns of substrate utilization with DTNB, lipoic acid, and selenite are identical to that of Sec-containing TR3 based upon both specific activity measurements and steady-state kinetic analysis. The same data also show that these enzymes are dissimilar to Sec-containing TR1. These data support the hypothesis that the chemical mechanisms of DmTR, CeTR, PfTR, and TR3 are similar with respect to the utilization of small molecule substrates but are distinct from that of TR1. This hypothesis was further tested using selenocystine as a substrate with the data presented below.

The Reduction of Selenocystine Does Not Require a Sec Residue in TR3-like Enzymes. The utilization of selenocystine, the selenium analogue of cystine, as a substrate further illuminates the mechanistic differences between TR1 and the other TR3-like enzymes listed in Table 1. As previously reported, DmTR, CeTR, and the Sec → Cys mutant of TR3 reduce selenocystine with an efficiency that is approximately half that of TR1.[7,20] Here we report that PfTR, a type II TR, reduces selenocystine with a specific activity higher than that of TR1 (Table 1), supporting our previous view that a Sec residue does not confer broad substrate specificity to TR per se.[20] The data in Table 1 also show that TR3 and PfTR use a similar mechanistic strategy to reduce selenocystine, and this mechanism is different from that of TR1. This point is explained below.

For TR3 and PfTR, the reduction of selenocystine occurs in the absence of a Sec residue but requires both N- and C-terminal redox centers for catalysis to occur. This is evidenced by the fact that the Sec → Cys mutant of TR3 retains ~19% of the activity of the WT enzyme (compare enzymes **4** and **5** in Table 1) while PfTR, absent a Sec residue, reduces selenocystine with a specific activity higher than that of TR1. In addition, enzyme **7**, with only a partial C-terminal redox center and missing a Sec residue, retains 10% of the activity of the WT enzyme. Similarly, PfTR mutants with a partial C-terminal redox center, enzymes **12** and **13**, have 6 and 24% of the activity of the WT enzyme (enzyme **11**), respectively. The data in Table 2 show that selenocystine is reduced very inefficiently when the C-terminal redox center is absent in TR3, type Ib, and type II TRs.

The data presented above are in marked contrast to the situation for TR1, which requires *both* N- and C-terminal redox centers and a Sec residue for the reduction of selenocystine. The Sec → Cys mutant of TR1 retains only 2% of the specific activity of the WT enzyme, and the mutants that contain only a partial C-terminal redox center retain only 1% of the activity of the WT enzyme (compare enzymes **3** and **15** with enzyme **1**). These data highlight the mechanistic differences between TR1 and TR3-like enzymes.

Mutants of DmTR Containing D-Cys in the C-Terminal Redox Center Differentiate Path A from Paths B and C. To further illustrate our point about three distinct mechanistic pathways, we constructed mutants of DmTR in which we replaced each of the C-terminal L-Cys residues with its enantiomer, D-Cys. This created two new mutants that contain either D-Cys-L-Cys dyads or L-Cys-D-Cys dyads in the C-terminal redox center of DmTR. The rationale for this replacement is that a D-Cys for L-Cys substitution makes disulfide bond formation between the adjacent Cys residues much less likely and also results in a very rigid structure that cannot adopt *cis* amide geometry in the peptide bond between the two Cys residues (ref 37 and unpublished observations of E. L. Ruggles and R. J. Hondal).[b] The overall result is a C-terminal redox center that contains two sulfur atoms just like the WT enzyme but is impaired and can be considered to be only a partial redox center similar to the mutants listed in Table 1.

The results of insertion of D-Cys into DmTR are listed in Table 4. Replacement of either Cys residue of the dyad results in a significant loss of Trx reductase activity (compare enzymes **9A** and **9B** with enzyme **9** in Table 4) and is most likely due to an inability to utilize path A (Figure 1). In contrast, enzymes **9A** and **9B** can reduce DTNB and selenocystine because they are still able to utilize paths B and C, respectively.

Table 4. Comparison of the Activities of D-Cys-Containing TRs with Various Substrates[a]

Enzyme		Activity [mol of NADPH min^{-1} (mol of TR)$^{-1}$]		
		Trx (90 μM)	DTNB (1 mM)	Selenocystine (50 μM)
DmTR-SCCS	**9**	165 ± 2	80 ± 10	46 ± 13
DmTR-SC_DC_LS	**9A**	3.4 ± 0.2	100 ± 6	41 ± 8
DmTR-SC_LC_DS	**9B**	0.4 ± 0.1	80 ± 6	7.7 ± 0.4

[a]A subscript D or L indicates that the Cys residue had D or L stereochemistry, respectively.

The Use of a "Guiding Bar" in TR1 Can Explain the Different Mechanistic Pathways. Here we present our explanation for why TR1 is dependent upon Sec for reduction of all of the different types of substrates listed in Table 1 and why the TR3-like enzymes can utilize other mechanistic pathways not available to TR1 and are therefore less dependent upon the use of Sec for substrate utilization. This explanation reconciles our accumulated data above with the crystal structure of TR1 and the proposal that TR1 uses a guiding bar to align the flexible C-terminal tail containing the C-terminal Cys-Sec redox center.[26,27] In determining the structure of TR1, Becker and co-workers noted the presence of a guiding bar, consisting of Trp407′, Asn418′, and Asn419′, in human TR1 that functions to suppress random motions and hold the tail more tightly to the TR1 enzyme body through a collection of weak forces, including hydrogen bonding interactions.[c] Figure 2 shows a sequence alignment of amino acids 376−422 of human TR1, containing the region of the guiding bar, and comparing it with the same region of human TR3 and the other TR3-like enzymes in this study. As this alignment shows, the TR3-like enzymes are missing residues Trp407′, Asn418′, and Asn419′ that were proposed to be critical for holding the C-terminal tail of TR1 in one of three positions favoring interaction with the N-terminal dithiol or alternatively with Trx. The three positions, CI, CII, and CIII, were elucidated from three conformations and from the interactions of certain key residues; the unit cell is a trimer of dimers, and each dimer has been captured at a different point in catalysis. This observation, combined with the proposed function of the guiding bar, fits well with the available crystal structures of human TR1, rat TR1, mouse TR3, DmTR, and PfTR

dx.doi.org/10.1021/bi400651x | *Biochemistry* 2014, 53, 554−565

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HsTR1     PLEYGACGLSEEKAVEKFGEENIEVYHSYFWPLEWTIPSR-DNNKCY--- 422
RnTR1     PLEYGCCGLSEEKAVEKFGEENIEVYHSFFWPLEWTVPSR-DNNKCY--- 422
HsTR3     PLEYGCVGLSEEEAVARHGQEHVEVYHAHYKPLEFTVAG-RDASQCY--- 447
MmTR3     PLEYGCVGLSEEEAVALHGQEHVEVYHAYYKPLEFTVAD-RDASQCY--- 440
DmTR      PLEYACVGLSEEDAVKQFGADEIEVFHGYYKPTEFFIP--QKSVR-Y--- 412
CeTR3     PLELSTVGLTEEEAIQKHGEDSIEVFHSHFTPFEYVVPQNKDSGFCY--- 426
PfTR      PIEYGACGYSEEKAYELYGKSNVEVFLQEFNNLEISAVHRQKHIRAQKDE 449
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Figure 2. Sequence alignment of various TRs in the guiding bar region of Pro376−Tyr422. Critical amino acids Trp407, Asn418, and Asn419 are present in TR1 of human (HsTR1) and rat (RnTR1) enzymes, but not mitochondrial TR3 enzymes from human (HsTR3), mouse (MmTR3), fruit fly (DmTR), nematode worm (CeTR3), or *P. facliparum* (PfTR).

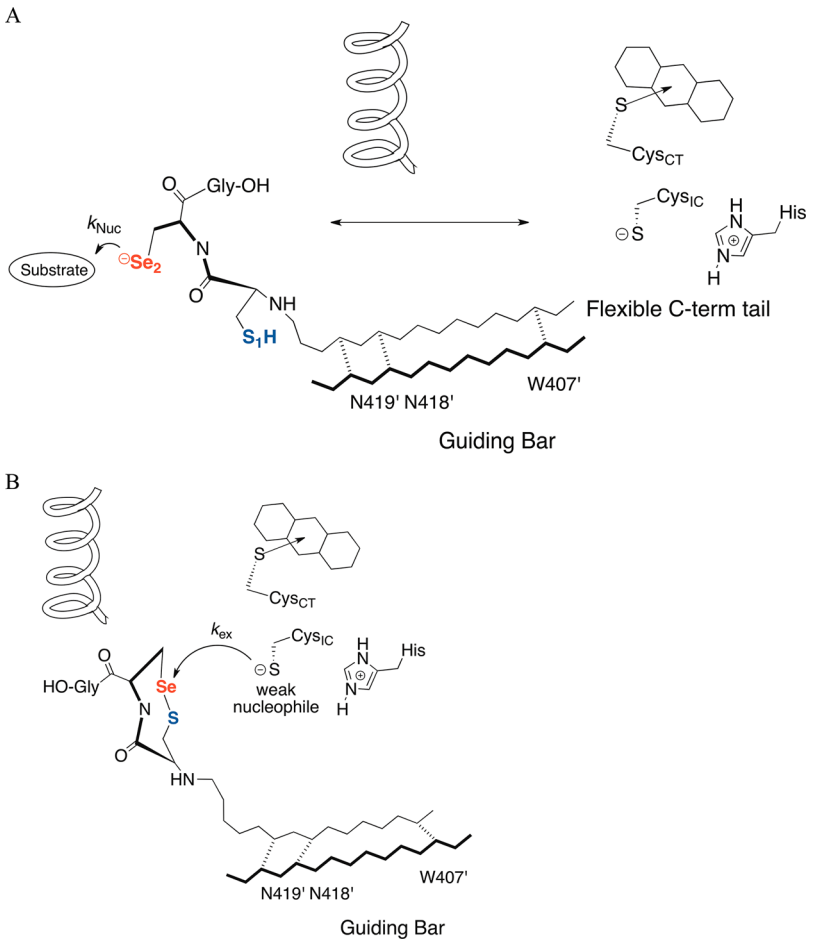

Figure 3. Trp407′, Asn418′, and Asn419′ in the guiding bar of TR1 suppress random motions of the C-terminal tail of the enzyme and restrict it to using path A (Figure 1) for the reduction of substrates. Because of this restricted motion, only the C-terminal redox center can donate electrons to the substrate (top), and only the C-terminal redox center can approach the N-terminal redox center to accept electrons (bottom). Most small molecule substrates would be excluded as a result. In this cartoon model, the Se atom is responsible for both donating electrons to the substrate and then accepting them from the N-terminal redox center. Replacement of the Se atom in the WT enzyme with a S atom in the Cys mutant would cause the rate to decrease in at least two ways in this model. First, a thiolate is less nucleophilic than a selenolate, and its use in the mutant would decrease the rate of nucleophilic attack on the disulfide bond of Trx (with associated rate constant k_{nuc}). Second, because a Se atom in a selenosulfide bond is much more electrophilic than a S atom in a disulfide bond, the thiol−disulfide exchange step between the N- and C-terminal redox centers (with associated rate constant k_{ex}) would be significantly slower. Because of the inability of TR1 to use path B or C, TR1 is very dependent upon the use of Se for the reduction of substrate molecules.

enzymes.[26−28,38−41] The C-terminal tail has been observed in crystal structures of the human[26,27] and rat[38] enzymes, but not mouse TR3,[40] PfTR,[41] or DmTR.[28] A possible explanation for this observation is that the absent guiding bar in mouse TR3, PfTR, and DmTR renders the C-terminal tails of these enzymes too mobile to generate sufficient electron density to observe the C-terminus of these enzymes. In contrast, the guiding bar present in the TR1 enzymes from human and rat limits the motion of the C-terminal tail, allowing it to be detected in the X-ray diffraction pattern.

As shown in the top panel of Figure 3, the more tightly held tail of the enzyme in TR1 allows only the C-terminal redox center to interact with substrates and prevents access to the N-terminal redox center by small molecule substrates. As depicted in Figure 3, the guiding bar restricts TR1 to exclusively use path A in Figure 1 for the reduction of substrates, making TR1 highly dependent upon the presence of a Sec residue in the C-

dx.doi.org/10.1021/bi400651x | *Biochemistry* 2014, 53, 554−565

terminus primarily because of both high selenolate nucleophilicity and *Se*-electrophilicity in a selenosulfide as described in the legend of Figure 3. This hypothesis is supported by experimental data provided by Becker and co-workers, who reported that the N419A mutant of TR1 had significantly increased activity toward DTNB, most likely because of the greater accessibility of the substrate to the N-terminal redox center, by impairing the ability of the guiding bar to hold the C-terminal tail rigidly.[27]

In contrast, as shown in Figure 4 the absence of Trp407′, Asn418′, and Asn419′ in the guiding bar of TR3-like enzymes

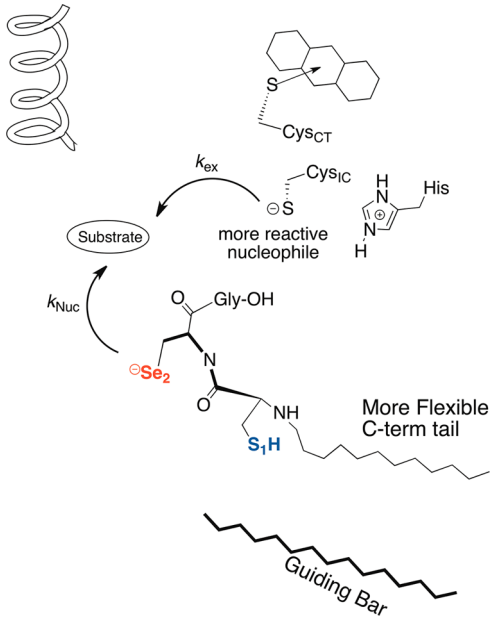

Figure 4. Trp407′, Asn418′, and Asn419′ in the guiding bar of TR3-like enzymes yield a much more flexible C-terminal tail and allows substrates to approach the N-terminal redox center. This additional flexibility allows TR3-like enzymes to utilize paths B and C in Figure 1 for the reduction of substrates. As a result, TR3-like enzymes are much less dependent upon the use of Se for substrate utilization.

allows much greater flexibility in the C-terminal tail of these enzymes. This affords TR3-like enzymes the ability to use paths B and C in addition to path A for substrate utilization (Figure 1), making them much less dependent upon the use of a Sec residue for the reduction of substrates.

The ability of TR3-like enzymes to use multiple catalytic pathways imbues them with the ability to reduce substrates in the absence of Sec due in part to greater access to the N-terminal redox center, but there are other factors at work, as well. Previous data from our laboratory indicate that the thiolate of Cys$_{IC}$ of TR3 is a weak nucleophile capable only of transferring electrons to very good electron acceptors. This is also most certainly true of Cys$_{IC}$ in TR1. However, our data here suggest that the N-terminal redox centers of TR3-like enzymes are chemically more reactive toward substrates such as DTNB than is the N-terminal redox center of TR1 (Table 1). This may be due to differences in the active site microenvironments of the respective enzymes that enhance the overall reactivity of the N-terminal redox center in TR3-like enzymes. Substrate discrimination through binding interactions and steric hindrance must also be considered as factors. This might help explain why TR1Δ2 (enzyme **15**) can reduce juglone[42] (containing an electrophilic α,β-unsaturated ketone group) and nitroaromatic compounds,[43,44] with activity nearly equivalent to that of the full-length WT enzyme, but reduces DTNB and lipoic acid very poorly, which are both highly electrophilic disulfides, because of the presence of electron-withdrawing groups (DTNB) and ring strain (lipoic acid).

Comparison of Selenocystine to Cystine as a Substrate. As explained in the legend of Figure 3, the guiding bar mostly restricts TR1 to utilize path A for the reduction of substrates. The use of this pathway makes TR1 highly dependent upon Se because substitution with S impairs the ability of the C-terminal redox center to donate electrons to the substrate and accept them from the N-terminal redox center. Are both of these factors equally important, or is one of them a dominating feature of the use of Se in TR? Traditionally, it has been assumed that *Se*-nucleophilicity is the dominating factor,[19,21] but the contribution of *Se*-electrophilicity should be considered, as well. The use of selenocystine as a substrate is instructive in this regard. Upon examination of the data in Table 1 with selenocystine as the substrate for the mutants of TR3 and PfTR with partial C-terminal redox centers (enzymes **6**, **7**, **12**, and **13**), it appears that path A is the optimal pathway for reduction of selenocystine for both enzymes because these variants are still able to reduce selenocystine, albeit at diminished rates. The use of this pathway results in an oxidized C-terminal redox center (irrespective of the presence of a Sec residue in the case of PfTR), which most likely is the optimal substrate for the N-terminal redox center in each respective enzyme. The use of path C by these mutants for the reduction of selenocystine allows for significant catalytic activity, and this occurs in the absence of a nucleophilic Sec residue. If cystine, with a S−S bond, is used as the substrate, there is little catalytic activity (data not shown). The difference in catalytic activities must be due to chemical differences between a substrate Se−Se bond and a substrate S−S bond. An obvious difference is the high electrophilic character of the Se−X$_2$ bond formed during the catalytic cycle of path C (middle portion of the bottom panel of Figure 1).

On the basis of our overall analysis and discussion in this paper, we suggest three reasons why TR1 is so dependent upon the use of Sec for the reduction of substrates. (i) The presence of the guiding bar in TR1 allows for the use of only path A, and this path allows only the oxidized C-terminal redox center to approach the N-terminal redox center for reduction to the exclusion of most other substrates. (ii) The apparent low chemical reactivity of the N-terminal redox center of the TR1 enzyme and the comparative analysis of selenocystine and cystine substrate utilization in TR3 described above suggest that *Se*-electrophilicty is required to accelerate the transfer of electrons from the N-terminal redox center to the Se atom because in its absence electron transfer is very slow. (iii) *Se*-nucleophilicity helps accelerate the transfer of electrons to the substrate.

When utilizing path A, Cys orthologs must compensate for the loss of both *Se*-nucleophilicity and *Se*-electrophilicity to accomplish the reduction of Trx. While some earlier work has addressed increased *S*-nucleophilicity in Cys orthologs,[19] we will address the issue of *S*-electrophilicity in the future.

A Hypothesis about SecTRAPs. It has been proposed that the guiding bar of TR1 functions to help prevent the leakage of electrons from the N-terminal redox center.[27] Such leakage is known to have deleterious effects. Arnér and co-workers have described TR1 enzymes in which the Sec residue has been electrophilically modified as "SecTRAPS".[42] SecTRAPS are

characterized by a gain of function by the N-terminal redox center. We propose that a Sec residue modified by an electrophile, as has been shown with electrophilic prostaglandins,[45] causes TR1 to undergo a conformational change that allows greater accessibility of the substrate to the N-terminal redox center (Figure 5). This would explain the gain of

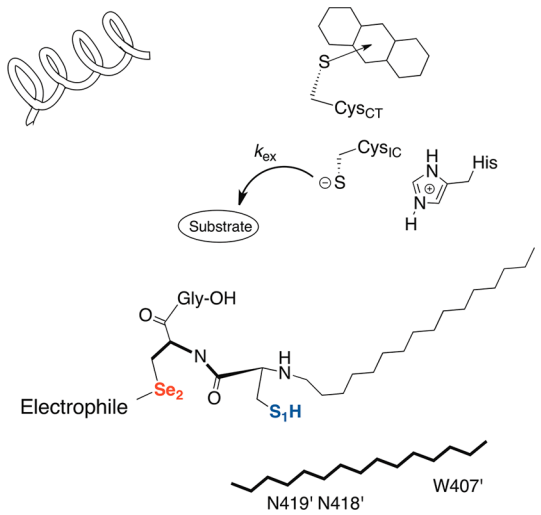

Figure 5. Hypothesis regarding SecTRAPs. SecTRAPS are characterized by a gain of function by the N-terminal redox center. Our hypothesis is that electrophilic modification of the Se atom results in a conformational change in TR1 allowing the enzyme the use of either path B or path C for the reduction of substrates. This could explain the cell killing ability reported for SecTRAPs.[42]

function property of SecTRAP enzymes and could explain their deleterious effects, possibly via the increased NADPH oxidase activity as reported by Arnér and co-workers.[42]

CONCLUSION

We have presented evidence that TR1 is kinetically and mechanistically distinct from TR3 and the TR3-like enzymes in this study. TR1 is highly dependent upon the use of a Sec residue because the amino acids of the so-called guiding bar restrict the movement of the C-terminal tail of the enzyme containing the Cys-Sec redox dyad. We propose that this restrictive motion permits the oxidized, C-terminal Cys-Sec dyad to interact with the N-terminal redox center only during the exchange step of the catalytic cycle. Selenium accelerates the rate of the reaction by acting as both a good acceptor of electrons during the exchange step and a good electron donor to the substrate during the nucleophilic attack step. Replacement of the selenium atom with sulfur therefore greatly slows the rate of the reaction because of the loss of *Se*-electrophilicity and *Se*-nucleophilicity. In contrast, TR3-like enzymes lack the guiding bar and can use alternative mechanistic pathways for the reduction of substrates and are therefore less dependent upon the use of a Sec residue for catalysis of many small molecule substrates. Cys orthologs must compensate for the absence of Sec by increasing *S*-nucleophilicity, *S*-electrophilicity, or both to be effective catalysts.

ASSOCIATED CONTENT

Supporting Information

Concentrations of enzymes and substrates used in the enzyme assays in this study (Tables S1 and S2, respectively). This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

These studies were supported by National Institutes of Health (NIH) Grant GM094172 to R.J.H. A.P.L. was supported by NIH Training Grant T32 HL07594 administered by Dr. Kenneth G. Mann.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge the Vermont Cancer Center DNA Facility for sequencing services. We are extremely grateful to Professor Charles Williams of The University of Michigan (Ann Arbor, MI) for a critical reading of this manuscript prior to submission.

ABBREVIATIONS

Ala, alanine; Asn, asparagine; βME, 2-mercaptoethanol; CeTR3, mitochondrial TR from *C. elegans*; Cys, cysteine; D-Cys, D-enantiomer of Cys; DmTR, TR from *D. melanogaster*; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; Gly, glycine; GR, glutathione reductase; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; L-Cys, L-enantiomer of Cys; LipDH, lipoamide dehydrogenase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight spectroscopy; M_r, molecular ratio; mTR3, mitochondrial TR from mouse; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced; PfTR, TR from *P. falciparum*; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Sec, selenocysteine; Ser, serine; SM, small molecule; TB, terrific broth; TGR, thioredoxin/glutathione reductase; TR, thioredoxin reductase; Tris, tris(hydroxymethyl)aminomethane; Trp, tryptophan; Trx, thioredoxin; U, one-letter code for Sec; WT, wild type.

ADDITIONAL NOTES

[a]Throughout this work, we use a nomenclature of TR-$AA_1AA_2AA_3AA_4$ to denote the source of the enzyme and its C-terminal tetrapeptide sequence. In addition, we use the nomenclature TRΔ2, TRΔ3, etc., to denote the number of C-terminal amino acids missing from the truncated enzyme.
[b]Please see Figure 4 from ref 28 and Figure 1 from ref 20 for a description of how the Cys-Cys dyad might adapt *cis* amide geometry during the catalytic cycle.
[c]The "prime" designation denotes amino acids that are part of the B chain and is used to distinguish residues from those of the A chain. The N-terminal and C-terminal redox centers are on opposing subunits.

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