Reaction Mechanism and Regulation of Mammalian Thioredoxin/Glutathione Reductase†

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ABSTRACT: Thioredoxin/glutathione reductase (TGR) is a recently discovered member of the selenoprotein thioredoxin reductase family in mammals. In contrast to two other mammalian thioredoxin reductases, it contains an N-terminal glutaredoxin domain and exhibits a wide spectrum of enzyme activities. To elucidate the reaction mechanism and regulation of TGR, we prepared a recombinant mouse TGR in the selenoprotein form as well as various mutants and individual domains of this enzyme. Using these proteins, we showed that the glutaredoxin and thioredoxin reductase domains of TGR could independently catalyze reactions normally associated with each domain. The glutaredoxin domain is a monothiol glutaredoxin containing a CxxS motif at the active site, which could receive electrons from either the thioredoxin reductase domain of TGR or thioredoxin reductase 1. We also found that the C-terminal penultimate selenocysteine was required for transfer of reducing equivalents from the thiol/disulfide active site of TGR to the glutaredoxin domain. Thus, the physiologically relevant NADPH-dependent activities of TGR were dependent on this residue. In addition, we examined the effects of selenium levels in the diet and perturbations in selenocysteine tRNA function on TGR biosynthesis and found that expression of this protein was regulated by both selenium and tRNA status in liver, but was more resistant to this regulation in testes.

Animal thioredoxin reductases (TRs)[1] are NADPH-dependent, FAD-containing proteins that belong to a pyridine nucleotide disulfide oxidoreductase family (*1*, *2*). To date, three thioredoxin reductases have been identified in mammals, including TR1 (the cytosolic TR, also called TrxR1, TxnRd1, or TrxRα), TR3 (mitochondrial TR, also called TrxR2, TxnRd2, or TrxRβ), and TGR (thioredoxin and glutathione reductase, also called TR2). TR1 and TR3 are the major thioredoxin reductases in the cytosol and mitochondria, respectively, and are ubiquitously expressed in various tissues and cell types. The catalytic mechanism of TR1 has been well-characterized, but there is little information about the reactions catalyzed by TR3 and TGR. Recently, TR1 and TR3 were shown to be essential for mouse embryogenesis (*3*, *4*), although through different mechanisms. TR1 was found to be critical for cell growth (*4*), whereas TR3 was shown to be essential for heart development (*3*).

Selenium is an essential trace element with significant cancer prevention potential and numerous other roles in human health (*5*, *6*). In cells, it is present in the form of (i) small molecular weight selenium compounds, (ii) a component of certain bacterial tRNAs, (iii) a protein cofactor, and (iv) selenocysteine (Sec) residues in proteins (*7*). Among these forms, Sec is by far the major functional form of selenium (*8*). This residue is inserted into proteins in response to UGA codons. UGA is a conventional stop signal, but in the presence of cis-acting Sec insertion sequence (SECIS) elements (*9*, *10*), it can be recoded to insert Sec. In addition to SECIS elements, trans-acting factors such as the Sec-specific elongation factor, Sec tRNA, and SECIS-binding proteins are involved (*11*−*16*). Sec tRNA ($tRNA^{[Ser]Sec}$) gene knockout is embryonic lethal in mice (*17*, *18*), consistent with the important role of Sec in biology. Mammalian thioredoxin reductases are selenoproteins, which contain a penultimate Sec in their C-terminal active center (*19*, *20*). Sec is essential for TR1 activity and function.

Several selenium deficiency mouse models, in which a mutant $tRNA^{[Ser]Sec}$ that lacks the highly modified isopentenyladenosine base at position 37 (designated $i^6A^-tRNA^{[Ser]Sec}$) was overexpressed in either wild-type (*21*, *22*) or $tRNA^{[Ser]Sec}$ knockout (*23*) genetic backgrounds, have been established to study the properties, functions, and hierarchy of selenoproteins. Both TR1 and TR3 were shown to have high priority for selenium supply, the finding that distinguished these proteins from stress-related selenoproteins, such as glutathione peroxidase 1 (*23*).

The recently identified TGR is unusual among TRs in that it has an additional N-terminal glutaredoxin (Grx) domain, which is fused to a canonical TR module (*24*). A similar

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EST, expressed sequence tag; HED, β-hydroxyethyl disulfide; IPTG, isopropyl β-D-thiogalactopyranoside; GPx, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; ORF, open reading frame; Sec, selenocysteine; SECIS, selenocysteine insertion sequence; TGR, thioredoxin/glutathione reductase; TR, thioredoxin reductase; Trx, thioredoxin.

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domain organization has recently been found in one of the human TR1 forms (*25*, *26*), as well as in several TRs from parasitic organisms (*27*−*29*). Mammalian TGR exhibits broad substrate specificity and can reduce various components of both thioredoxin and glutathione systems (*24*). A three-dimensional molecular structure of TGR has been modeled in silico, and a reaction mechanism has been proposed (*24*). We also identified a disulfide bond isomerization activity of this protein and suggested its role in formation of structural components of sperm (*30*).

In this report, we developed various recombinant TGR forms and used them for structure/functional studies of this enzyme. These experiments demonstrated the key role of Sec and supported the previously proposed reaction mechanism of TGR (*24*). In addition, expression analysis of TGR under various conditions that perturb selenium homeostasis showed that TGR was regulated by these conditions in liver, but it was largely immune to this regulation in testes.

MATERIALS AND METHODS

Analysis of Thioredoxin Reductase Distribution. NCBI nonredundant and EST databases were analyzed by tBLASTN using human TR1 as query. The identified sequences were aligned with three human TRs and grouped by phylogenetic analysis using PHYLIP in BioEdit. Phylogenetic analyses were carried out separately on entire protein sequences and truncated sequences common to all hits.

Preparation of Recombinant Mouse TGR and Its Mutant Forms. Wild-type recombinant mouse TGR and its mutant forms (Sec614Cys, Sec614Ser, and a form truncated at Cys613) were produced as recombinant proteins expressed in *Escherichia coli*. To generate the constructs, the open reading frame (ORF) of mouse TGR (*24*) was amplified by PCR and ligated at the introduced *Nde*I and *Eco*RI sites into pET21(b) vector (Novagen). 5′-GAGATTCCATATGGCGTCGCCACCCGGCCGCCG-3′ was used as the forward primer for the wild-type and mutant TGR forms. For the wild-type form, 5′-CGGAATTCTTAGCTAGCGATTGGTGCAGACCTGCAACCGATGGCTAGCCTCAGCAGCCTTTCTGAG-3′ was used as the reverse primer. This sequence contains a bacterial SECIS element, derived from the *E. coli* formate dehydrogenase H gene, immediately downstream of the TAG stop signal. 5′-CGGAATTCCTAGCCGCAGCAGCCTTTCTGAG-3′, 5′-CGGAATTCCTAGCCTGAGCAGCCTTTCTGAG-3′, and 5′-CGGAATTCCTAGCCTTAGCAGCCTTTCTGAG-3′ were used as reverse primers for the Sec613Cys mutant, the Sec614Ser mutant, and the truncated form, respectively.

The resulting constructs were transformed into *E. coli* BL21 (DE3) cells (Novagen). Cells were grown in LB medium with ampicillin at 37 °C until the absorbance at 600 nm was above 0.5, followed by changing the temperature to 15 °C. Thirty minutes later, 5 μM sodium selenite was added to the medium. After an additional 30 min, 50 μM IPTG was added and cells were grown further for 18−24 h. Cells were then centrifuged and proteins isolated according to the pET System Manual (Novagen) and the QIAexpressionist (QIAGEN).

We also generated TGR constructs expressing proteins containing GST- or His-tags. pET 28(a) vector (Novagen) was used for the His-tag constructs, and pGEX4T1 vector (Amersham Biosciences) was used to prepare the GST-tag constructs. To express proteins, cells carrying the plasmids were induced with 1 mM IPTG and, after 3−4 h of protein synthesis at 37 °C, were collected. In addition, the TGR construct coding for the His-tagged protein was cotransformed into *E. coli* BL21 (DE3) cells with a pG-Tf2 construct, which encodes a trigger factor and GroEL-GroES chaperones (*31*), and protein synthesis was induced as described above.

Constructs encoding Grx and TR domains of TGR with either His-tag or GST-tag were also generated. For the Grx domain construct, 5′-CGGAATTCTTAGTCATCTTGAAGGAGCTTCTGCA-3′ was used as the reverse primer. This construct encoded a 123-amino acid protein corresponding to the N-terminal sequence of TGR, including the Grx active center and the GSH binding motif. For the TR domain construct, 5′-GAGATTCCATATGTCGGCTCATGATTACGACCTCAT-3′ was used as the forward primer. It encoded a 492-amino acid protein corresponding to the TR domain of TGR. It included the pyridine nucleotide disulfide oxidoreductase portion and the C-terminal extension but lacked the Grx domain.

^{75}Se Labeling of Recombinant TGR Forms. To label cells with ^{75}Se, cells carrying plasmids were grown at 37 °C until the OD_{600} reached 0.5−0.7 and ∼20 μCi ^{75}Se (as freshly neutralized sodium selenite, specific radioactivity ∼1000 Ci/mmol, University of Missouri Research Reactor) was added in addition to 5 μM sodium selenite. After an additional 30 min, 50 μM IPTG was added to each cell culture. After 18−24 h of protein synthesis at 15 °C, cells were collected, sonicated, and centrifuged at 18 000*g* for 30 min. Cell lysates were then subjected to SDS−PAGE followed by transfer of proteins onto a PVDF membrane. ^{75}Se signal was visualized with a PhosphorImager.

Isolation of TR1, TGR and Recombinant TGR, Grx Domain, and Grx2. Mouse and rat TR1 and TGR were isolated from liver and testes (Pel-Freez Biologicals, Rogers, AR) using a three-step procedure that included anion-exchange chromatography on a DEAE-Sepharose column, affinity chromatography on an ADP-Sepharose column (Amersham Biosciences), and hydrophobic interaction chromatography on a phenyl-HPLC column (*32*, *33*). Recombinant TGR forms were also isolated from *E. coli* according to this procedure. His-tagged TGR forms and the Grx domain were isolated using a Ni-NTA resin (Novagen) and ADP-Sepharose according to the corresponding product manuals. Recombinant human Grx2 protein was isolated as described previously (*34*).

Analysis of Selenium Content. Selenium content of the recombinant wild-type TGR was analyzed by Inductively Coupled Plasma-Emission Spectrometry (ICP) at the Chemical Analysis Facility at University of Georgia.

Molecular Modeling of TGR. The TR domain of TGR was modeled using SWISS-MODEL (*35*) based on the crystal structure of rat TR1 (*36*) (PDB number 1H6V); similarly, the Grx domain was separately modeled based on an *E. coli* Grx structure (*37*) (PDB number 3GRX). The two domains were manually fit in DeepView (*35*). The figure illustrating the active site was generated using Chimera (*38*).

Western Blotting. Samples were subjected to SDS−PAGE, and the separated proteins were electrophoretically transferred to a PVDF membrane. The membrane was blocked with 5%

dry fatfree milk in phosphate-buffered saline (PBS, 10 mM sodium phosphate and 0.15 M NaCl, pH 7.4) containing 0.1% Tween 20 (PBST) for 1 h at room temperature. Primary antibodies (1:1000 dilution) in PBST with 5% milk were added and incubated with the membrane either for 1 h at room temperature or overnight at 4 °C. After washing with PBST, the membrane was incubated with secondary antibody (1:3000 dilution, 5% milk in PBST). The membrane was then washed again with PBST and developed with the ECL system (Amersham Biosciences).

Enzyme Assays. TR activities of TR1 and TGR were assayed by two methods. (i) NADPH-dependent reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was determined as the increase in absorbance at 412 nm at 25 °C (*39*). The reaction mixture contained 0.2 mM NADPH, 5 mM DTNB, and 10 mM EDTA in 0.5 mL 100 mM phosphate buffer, pH 7.0. (ii) NADPH-dependent reduction of 3 μM *E. coli* thioredoxin (Trx) and 0.5 mg/mL insulin was determined as the decrease in absorbance at 340 nm at 25 °C (*39*). The reaction mixture contained 0.2 mM NADPH, 0.5 mg/mL insulin, 1 mM EDTA, and 3 μM Trx in 0.5 mL 50 mM phosphate buffer, pH 7.0.

Glutathione reductase (GR) activity of TGR was assayed as NADPH-dependent reduction of oxidized glutathione (GSSG) determined as the decrease in absorbance at 340 nm at 25 °C (*40*). The reaction mixture contained 0.2 mM NADPH, 5 mM GSSG, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.6, in 0.5 mL. Grx activity was assayed as the decrease in absorption at 340 nm at 25 °C. The reaction mixture contained 0.2 mM NADPH, 1 mM GSH, 2 mM EDTA, 0.4 units of GR, 1 mM β-hydroxyethyl disulfide (HED), and 0.1 M Tris-HCl buffer, pH 8.0, in 0.5 mL. To determine kinetic parameters of the Grx domain, substrate concentrations were 50−500 μM GSH and 100−1000 μM HED. Lineweaver−Burk plots were used to determine apparent K_m and k_{cat} values (*41*).

Transgenic Mice, Rescue Mice, Dietary Supplementation, Tissue Sample Preparation, and ^{75}Se Labeling of Animals. Transgenic mice overexpressing i^{6}A$^-$Sec tRNA$^{[Ser]Sec}$ used in this study were previously described (*22*). Selenium diets were purchased from Harlan Teklad (Madison, WI). Preparation of animal tissue samples and labeling of mice with ^{75}Se were performed as previously described (*22*). Briefly, following labeling of an animal with 0.3−0.4 mCi of ^{75}Se per animal for 48 h, tissues were collected, proteins extracted, resolved by SDS−PAGE, and transferred onto a PVDF membrane. Radioactive bands were detected using a PhosphorImager. In the ^{75}Se labeling experiments, we found that the labeling of selenoproteins in the selenium-deficient samples was stronger than that in selenium-sufficient samples. This is likely because of dilution of ^{75}Se radioactivity by nonradioactive selenium present in selenium-sufficient animals. The rescue mice, in which the tRNA$^{[Ser]Sec}$ was disrupted and the lethality rescued by expression of i^{6}A$^-$Sec tRNA$^{[Ser]Sec}$ transgene, were described in a recent study (*23*).

RESULTS

Distribution of Thioredoxin Reductases. We computationally analyzed three mammalian thioredoxin reductase sequences (TR1, TGR, and TR3) for occurrence in animal genomes (Table 1). We detected human, chimpanzee, mouse, rat, cow, and frog TGR sequences. TGR was also detected in chicken, indicating that this protein is not unique to mammals. In some mammals (pigs and dogs), no TGR genes were found, probably due to incomplete genome sequences and lack of ESTs. In zebrafish, TGR and TR3 were detected, whereas TR1 was not detected either in EST or completed genome sequences (Table 1). The zebrafish TGR clustered with the frog TGR rather than with mammalian TR1 in the phylogenetic tree (data not shown). A large number of available ESTs for zebrafish TGR suggest that TGR is the dominant TR form in this organism. Apparently, this is also the case in other fishes, such as fugu and *Tetraodon nigroviridis*, whose genomes have been sequenced. Overall, these data suggested that, in organisms containing TGR and TR1, the latter protein is the major thioredoxin reductase, whereas TGR may serve a specialized function. In contrast, in organisms containing TGR and lacking TR1, TGR likely serves the function of both proteins. This is consistent with the finding that parasitic TGR homologues present in *Echinococcus granulosus* and *Taenia crassiceps* may account for the entire thioredoxin reductase activity in these organisms (*27*, *29*).

Table 1: Distribution of Thioredoxin Reductases in Humans and Animal Model Organisms

	TR1	TGR	TR3
human (*Homo sapiens*)	+	+	+
chimpanzee (*Pan troglodytes*)	+	+	+
mouse (*Mus musculus*)	+	+	+
rat (*Rattus norvegicus*)	+	+	+
pig (*Sus scrofa*)	+	ND[a]	+
cow (*Bos taurus*)	+	+	+
dog (*Canis familiaris*)	+	ND[a]	+
chicken (*Gallus gallus)*	+	+	ND[a]
nematode (*Caenorhabditis elegans)*	+	ND[a]	+
fruit fly (*Drosophila melanogaster*)	+	ND[a]	+
frog (*Xenopus laevis*)	+	+	+
zebrafish (*Danio rerio*)	-	+	+
fugu fish (*Takifugu rubripes)*	-	+	+
Tetraodon nigroviridis	-	+	+

[a] ND: not detected.

In contrast to TGR and TR1, which appeared to recently evolve from a common ancestor, TR3 is a more distantly related protein. Most members of the TR3 subfamily have predicted mitochondrial signals. Thus, their functions are probably associated with the mitochondrial function, although alternatively spliced cytosolic forms of this protein were predicted (*42*−*44*) and demonstrated (Turanov and Gladyshev, unpublished data).

Preparation of Recombinant TGR Forms. In a previous study, we proposed a structural model of TGR (*24*), in which the Sec was located between the thiol/disulfide active site in the TR portion and the redox active center of the Grx domain of the enzyme. To test this model, we generated a recombinant mouse TGR expressed in *E. coli*. Although eukaryotic and bacterial Sec insertion systems are incompatible, the presence of Sec near the C-terminus allowed us to employ the bacterial Sec insertion system for expression of Sec-containing forms of TGR by introducing the *E. coli* formate dehydrogenase H SECIS element immediately downstream of the stop signal in the TGR gene (*45*, *46*) (Figure 1). This method allows preparation of substantial quantities of TR1 (*47*) but has not been tested with other thioredoxin reductases.

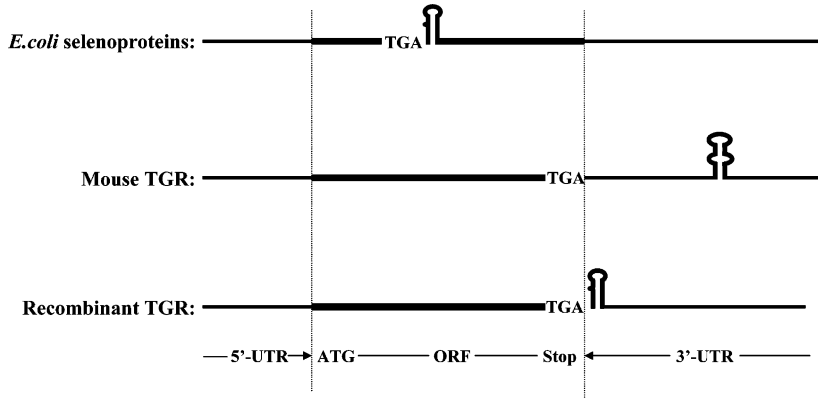

FIGURE 1: Schematic illustration of the construct for expression of selenoprotein form of TGR. *E. coli* selenoproteins contain an *E. coli* SECIS element, which is located within open reading frames (ORFs) immediately downstream of UGA (upper construct). Mouse TGR contains a different SECIS element, which is located in the 3′-untranslated region (3′-UTR) (middle construct). We took advantage of the C-terminal penultimate location of Sec in TGR and cloned *E. coli* formate dehydrogenase H SECIS element immediately downstream of the mouse TGR ORF (lower construct). The latter construct was used for expression of the Sec-containing TGR in *E. coli* cells.

We found that the recombinant TGR was mostly insoluble when expressed in *E. coli*, and neither cloning of the enzyme that contained various tags nor coexpression of TGR with chaperones increased its solubility (Supporting Information, Figure S1). However, we were able to obtain small amounts of the soluble enzyme by purifying it from a large quantity of cells, in which protein synthesis was induced slowly for a period of 24 h at a low temperature (see Materials and Methods for details). We obtained pure TGR using a two-step affinity chromatography procedure that included protein isolation on a His-tag resin and ADP-Sepharose (Supporting Information, Figure S1). Besides the wild-type TGR, we produced three mutants: Sec614Cys, Sec614Ser, and a truncated form with the C-terminal Sec−Gly dipeptide deleted. All these mutant proteins were expressed and purified similarly to the wild-type recombinant protein. These recombinant TGR forms and the native TGR that was directly purified from mouse testes exhibited similar physicochemical characteristics (Figure 2), except that the purified testis TGR lacked seven N-terminal residues MSSPPGR (compared to the recombinant proteins) as determined by protein sequencing by Edman degradation (data not shown). We also generated nontagged recombinant TGR proteins, and these proteins displayed similar characteristics as the tagged proteins.

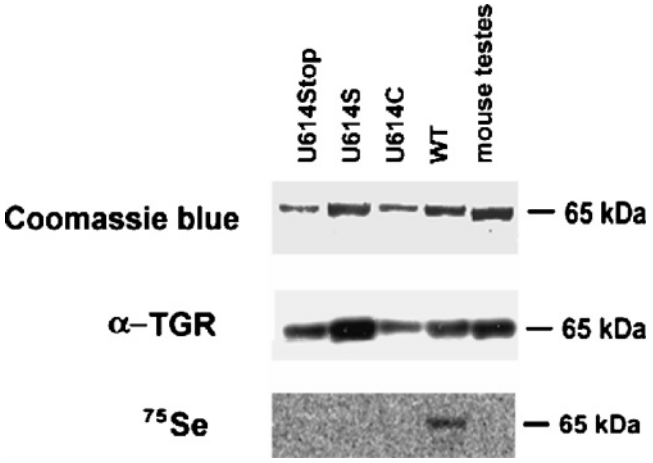

FIGURE 2: Characterization of recombinant TGR forms. Recombinant wild-type (designated as WT), Sec614Cys (U614C), Sec614Ser (U614S), and truncated forms of TGRs (U614Stop) were overexpressed in *E. coli* and purified. To label TGR with ^{75}Se, protein synthesis was induced in the presence of [^{75}Se]selenite. These recombinant proteins and TGR isolated from mouse testes were subjected to SDS−PAGE and Coomassie blue staining (upper panel), immunoblot analysis with antibodies specific for TGR (middle panel), and ^{75}Se detection by PhosphorImager analysis (lower panel). Approximate mass weight of one subunit of TGR is shown on the right.

The recombinant wild-type TGR was ^{75}Se-labeled when the bacterial growth medium was supplemented with ^{75}Se-[selenite] (Figure 2), indicating that the *E. coli* SECIS element was functional and allowed insertion of Sec in response to the UGA codon. Analysis of the selenium content of the recombinant wild-type TGR revealed that the enzyme had 0.1 equiv of selenium. Thus, this protein was likely a ∼1:9 mixture of the full-size selenoprotein and the protein truncated at Cys613. Sec insertion into recombinant proteins is known to be inefficient in *E. coli* (*48*). The relatively high proportion of Sec in our protein preparations compared to that in other TRs prepared under similar conditions was likely due to slow protein synthesis conditions employed in our study, which allowed more efficient utilization of the cellular Sec insertion machinery.

Role of Sec in TGR Activities. Recombinant TGR forms were further characterized with respect to TR, GR, and Grx activities (Table 2 and Figure 3). Both tagged and nontagged proteins showed similar activities (Tables 2 and 3 show the activities of the tagged proteins). There was only some decrease in TR activity in mutant TGRs compared to the wild-type recombinant form when DTNB and NADPH were used as substrates (Table 2, Figure 3). However, in the Trx/insulin assay, Sec614Ser and truncated forms were inactive, and the Sec614Cys preparation had 21% activity compared to that of recombinant wild-type TGR. This was equivalent to ∼2% activity of the Cys mutant taking into account that only ∼10% of the wild-type enzyme contained Sec and was active. Thus, the C-terminal penultimate Sec was required for the TR activity of TGR and the Cys residue could only partially compensate for the lack of Sec.

Similar to the TR activity, GR and Grx activities of wild-type and mutant TGRs required Sec: Sec614Cys TGR had 18% of the GR activity and 14% of the Grx activity compared to wild-type recombinant TGR (equivalent to 1.8% and 1.4% of wild-type activity taking into account the 10%

Table 2: Specific Activities of Recombinant TGR Forms[a]

protein form	DTNB reduction	Trx reduction	GR	Grx (without GSH)	Grx (with GSH)
native protein	3100 ± 350[b]	4200 ± 600[b]	2000 ± 340[b]	N/A	1900 ± 200[b]
wild-type	174 ± 26	40.0 ± 4.1	23.0 ± 1.5	61.2 ± 8.5	413 ± 42
Sec614Cys	123 ± 22	8.4 ± 1.4	4.2 ± 1.0	8.5 ± 3.3	348 ± 38.6
Sec614Ser	159 ± 26	0	0	0	388 ± 43.6
Sec614Stop	148 ± 24	0	0	0	429 ± 46.6
protein amount (μg)	15	150	150	100	15

[a] Activities are expressed in mU (nmol/(min·mg) of protein). [b] Data from ref *34*.

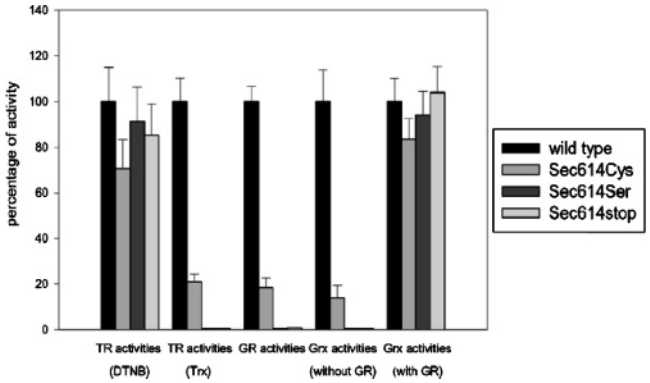

FIGURE 3: Catalytic activities of recombinant wild-type and mutant TGRs. Purified recombinant wild-type, Sec614Cys, Sec614Ser, and Sec614stop TGRs were tested for TR activities using DTNB and Trx assays, glutathione reductase (GR) activity, and Grx activities with/without addition of yeast GR as indicated in the figure. Activities of mutant TGR forms were calculated as the percentage of wild-type recombinant TGR activities.

Table 3: Kinetic Parameters of the Grx Domain of TGR

	Grx domain	human Grx2
specific activity (U/mg)	29.7	46.7
K_m for HED (μM)	399	1680[a]
k_{cat} for HED (min^{-1})	272	40[a]
K_m for GSH (μM)	317	ND[b]
k_{cat} for GSH (min^{-1})	231	ND[b]

[a] Data from ref *34*. [b] ND, not detected.

Sec insertion rate), while the Sec614Ser mutant and the truncated form had no detectable NADPH-dependent GR and Grx activities (Table 2, Figure 3). However, in the presence of yeast GR and glutathione, the Grx activities of both mutant TGRs (Sec614Ser and truncated form) were restored to the level of the wild-type enzyme (Table 2 and Figure 3). These findings suggested that (i) the Grx activity of TGR could be supported by either reduced glutathione or intramolecular electron transport, and (ii) the GR activity of TGR was dependent on the Sec residue. Like TRs, the proteins of the GR family are pyridine nucleotide disulfide oxidoreductases, but they lack the C-terminal extension containing the Sec-containing active center. These enzymes reduce glutathione by its thiol/disulfide active site, whereas our data suggest that it is the Grx domain of TGR that is responsible for the GR activity of this enzyme and that its regeneration is fully dependent on the reducing equivalents provided by Sec. Thus, Sec is a crucial player in all physiologically relevant activities of TGR. These data validate our previous structural model and the proposed reaction mechanism of TGR.

It is noteworthy that the activities of the recombinant wild-type TGR, when recalculated by taking into account the low content of selenium in this protein, were lower (by a factor of 10) than those of the native TGR purified from mouse testes. It is possible that (i) there were unknown post-translational modifications in the native protein, which resulted in optimal catalytic activities, whereas the recombinant proteins expressed in *E. coli* lacked these modifications and/or that (ii) the recombinant proteins were not fully folded and therefore not fully active. Nevertheless, the recombinant proteins had sufficiently high catalytic activities and showed substantial differences between wild-type and mutant forms to investigate the role of Sec as described above. In addition, consistent effects of the Sec mutation were observed for all activities, and the data were in good agreement with the known role of Sec in Trx reduction. We also prepared recombinant selenoprotein forms of two other mammalian thioredoxin reductases, TR1 and TR3 (ref *25* and unpublished data), and found that these enzymes were fully active when their activities were recalculated to their selenium content. Thus, the lower activity of the recombinant TGR was the property of this isozyme.

Kinetic Properties of the Grx Domain of TGR. Although kinetic characterization of TGR forms suggested that the Grx domain was catalytically competent, this domain had a CxxS sequence instead of the CxxC motif present in most glutaredoxins. To further examine its function, we characterized the recombinant Grx domain of the enzyme. Human Grx2 (*34*, *49*), the closest known homologue of the Grx domain, was used as a control. Grx2 had a fully functional CxxC motif (*34*). When HED was used as substrate, the specific activity of the Grx domain was 29.7 U/mg, which was similar to that of human Grx2, 46.7 U/mg (Table 3). K_m (399 and 317 μM) and k_{cat} (272 and 231 min^{-1}) values of the Grx domain for HED and glutathione, respectively, were also comparable to those of human Grx2 (Table 3) (*34*). In addition, we mixed the Grx domain or Grx2 with purified rat TR1 or TGR at different ratios and tested their GR activities. Figure 4 shows that Grx2 and the Grx domain increased GR activities of the mixtures and that the Grx domain was a better substrate than human Grx2 for both enzymes.

These data suggested that (i) the Grx domain in TGR was a functional glutaredoxin, although it lacked a canonical Grx active site, and (ii) the Grx domain had affinity for both thioredoxin reductases. Thus, this domain could catalytically interact with both major cellular redox systems (i.e., Trx and GSH systems), and together with the ability of Sec to transfer reducing equivalents to Grx and Trx, the Grx domain was responsible for the broad substrate specificity of TGR observed in in vitro assays.

TGR Expression in Mice Subjected to Selenium Deficiency and Perturbations in Sec tRNA Function. We previously developed a transgenic mice model to achieve selenoprotein-

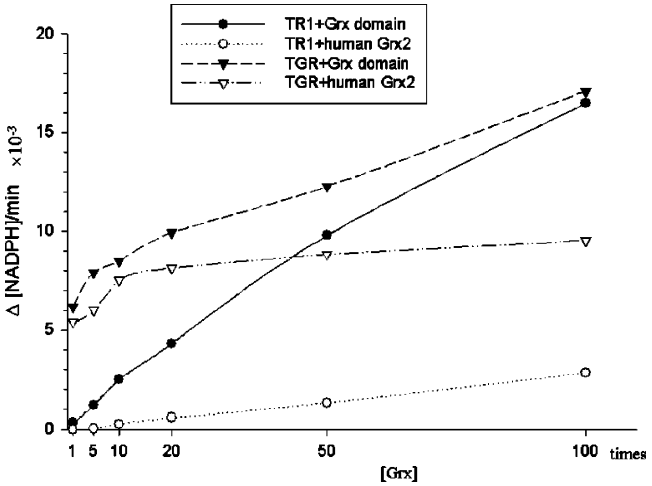

FIGURE 4: Dependence of glutathione reductase (GR) activity of TGR and TR1 on glutaredoxin 2 (Grx2) and glutaredoxin domain of TGR. Purified recombinant Grx domain of mouse TGR or human Grx2 was added to solutions of rat liver TR1 or testes TGR at different molar ratios (1:1 to 100:1, shown as ratio of glutaredoxin to thioredoxin reductase), followed by determination of GR activity of the mixtures.

specific deficiency by overexpressing a mutant Sec tRNA molecule (i^6A^-Sec $tRNA^{[Ser]Sec}$) (*22*). Here, we used this model to assess expression of TGR under conditions of normal and compromised selenium homeostasis. As TGR levels are low in most tissues (*30*), we enriched the protein on affinity columns in parallel for all samples to monitor TGR levels and then analyzed TGR by immunoblot assays. Using this procedure, we found that TGR levels were decreased in the livers of either i^6A^- transgenic mice maintained on a diet containing normal levels of selenium (Figure 5, compare lanes 1 and 3) or wild-type mice maintained on a Se-deficient diet (Figure 5, compare lanes 1 and 2). Dietary selenium regulated TGR levels to a greater degree than the presence of the i^6A^- transgene in the liver samples.

We also analyzed selenoprotein expression by metabolically labeling these proteins with ^{75}Se in mice. Comparison of the labeled selenoproteins from several tissues of wild-type and $i^6A^-tRNA^{[Ser]Sec}$ mice maintained on the Se-deficient diet for 1 month showed a decrease in glutathione peroxidase 1 (GPx1) expression, which is a major mammalian selenoprotein, whereas a strong ^{75}Se signal of TGR was retained in the testes (Figure 6).

Finally, we analyzed TGR expression in testes and liver of $i^6A^-tRNA^{[Ser]Sec}$ rescued mice, which lack the $tRNA^{[Ser]Sec}$ gene and the lethality is rescued by expression of the mutant $tRNA^{[Ser]Sec}$ transgene (*23*). In these mice, expression of stress-related selenoproteins (e.g., GPx1) is severely inhibited (Figure 7, Δtrsp-t-trspi$^6A^-$ lanes), whereas housekeeping selenoproteins that maintain the cellular redox status, such as TR1, are little affected. We found that the expression of TGR was only slightly decreased in testes of these mice compared to heterozygous mice and to mice rescued with the wild-type $tRNA^{[Ser]Sec}$ transgene (Figure 7A, left panel). To exclude the possibility that the truncated protein lacking the C-terminal Sec−Gly dipeptide was synthesized, we labeled these mice with ^{75}Se and found that the levels of ^{75}Se-labeled TGR (Figure 7A, left and middle panels) corresponded to those detected by western blot assays (Figure 7A, right panel). Thus, Sec could be efficiently inserted into TGR by the mutant Sec tRNA in testes.

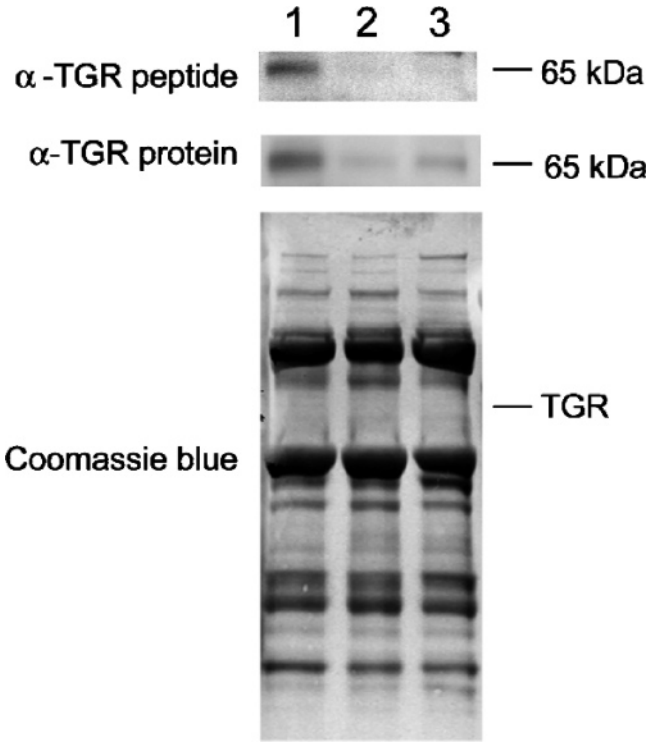

FIGURE 5: TGR expression analyses. Immunoblot analyses of TGR (top and middle panels showing assays with antibodies developed against the C-terminal peptide and the whole protein, respectively) in ADP-Sepharose-enriched fractions of mouse liver. Coomassie blue staining of the SDS−PAGE gel (bottom panel) is shown as protein loading control. The samples are as follows: lane 1, wild-type mouse maintained on a Se-sufficient diet; lane 2, wild-type mouse maintained on a Se-deficient diet; and lane 3, i^6A^- mutant Sec $tRNA^{[Ser]Sec}$ mouse maintained on a Se-sufficient diet. Migration of TGR is indicated on the right of the gels.

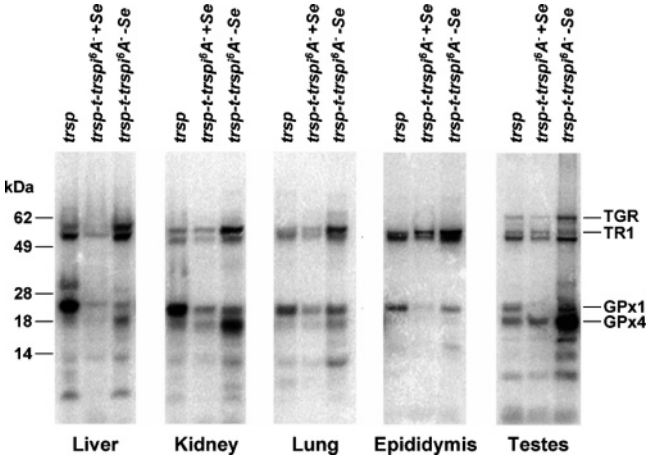

FIGURE 6: Selenoprotein expression in mice maintained on selenium-deficient and selenium-sufficient diets. Selenoprotein expression in indicated tissues was assessed by labeling mice with ^{75}Se, followed by SDS−PAGE analysis of protein extracts from each tissue and PhosphorImager detection of selenoproteins. This figure shows metabolic labeling of selenoproteins in liver, kidney, lung, epididymis, and testes samples of wild-type (trsp) and transgenic mice encoding 10 copies of the $i^6A^-tRNA^{[Ser]Sec}$ transgene (designated as trsp-t-trspi$^6A^-$) that were maintained on Se-sufficient (+Se) or Se-deficient (−Se) diets.

To analyze TGR expression in the livers of rescued and control mice, we again enriched the protein on ADP-

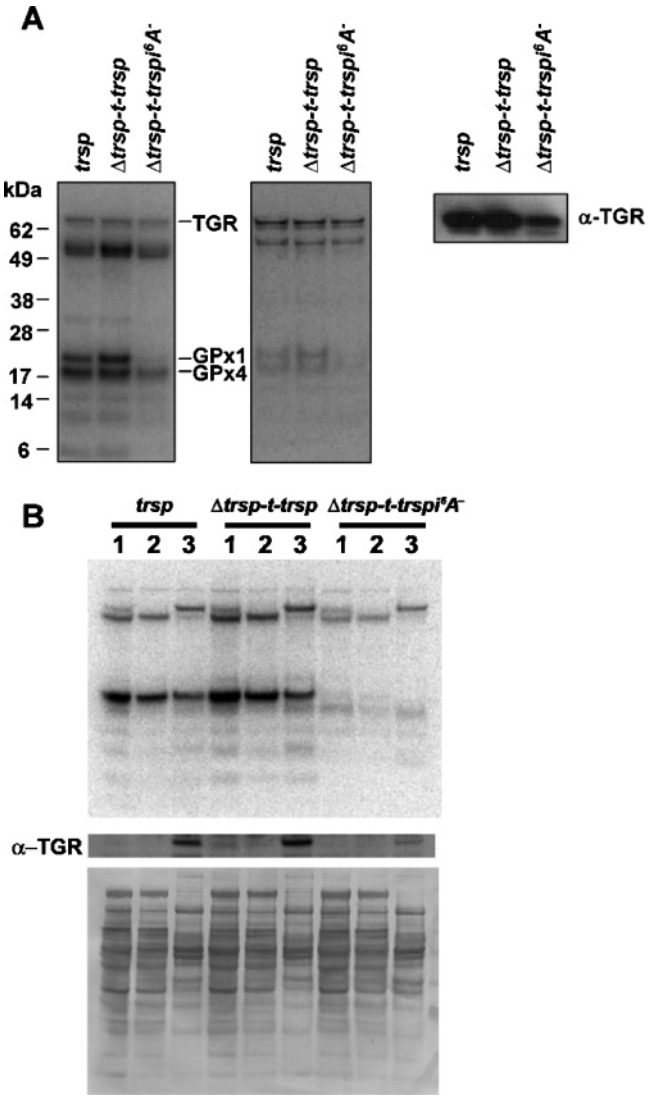

FIGURE 7: TGR expression in the $i^6A^-tRNA^{[Ser]Sec}$ rescued mouse. Expression of TGR in testes and livers of rescued and control mice was assessed by ^{75}Se-labeling of mice and western blot assays. (A) ^{75}Se-labeling and western blot analysis of testes samples. Left panel, PhosphorImager analysis of ^{75}Se-labeled testis extracts fractionated on an SDS−PAGE gel. Migration of TGR, GPx1, and GPx4 is indicated. Middle panel, each testis sample was enriched, in parallel, for TGR on ADP-Sepharose, and the enriched fractions were analyzed by SDS−PAGE and a PhosphorImager. Right panel, western blot of the enriched testis fractions with antibodies specific for TGR. (B) ^{75}Se-labeling and western blot analysis of liver samples. Top panel, PhosphorImager analysis of ^{75}Se-labeled liver extracts fractionated on ADP-Sepharose and resolved on an SDS−PAGE gel; middle panel, western blot of the same membrane using anti-TGR antibodies; lower panel, Coomassie blue staining of the samples shown in the two upper panels. Lane designation is as follows: 1, cell lysates; 2, ADP-Sepharose flow-through fractions; 3, samples eluted from ADP-Sepharose. Animal designation in panels A and B is as follows: trsp, wild-type mouse; Δtrsp-t-trsp, $tRNA^{[Ser]Sec}$ knockout/transgenic mouse lacking the endogenous $tRNA^{[Ser]Sec}$ gene and encoding 10 copies of the wild-type $tRNA^{[Ser]Sec}$ transgene; Δtrsp-t-trspi$^6A^-$, $tRNA^{[Ser]Sec}$ knockout/transgenic mouse lacking the endogenous $tRNA^{[Ser]Sec}$ gene and encoding 20 copies of the $i^6A^-tRNA^{[Ser]Sec}$ transgene.

Sepharose (Figure 7B). After the enrichment, TGR was detected in wild -type, heterozygous rescued, and homozygous rescued mice, suggesting that the mutant Sec tRNA could insert Sec into liver TGR. These data placed TGR in line with TR1, TR3, and GPx4 in regard to regulation of selenoprotein expression. However, we found that the TGR synthesis was affected to a greater degree in liver than in testis samples (Figure 7).

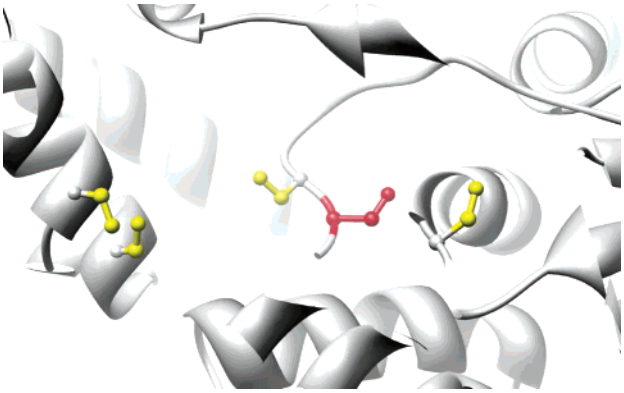

FIGURE 8: Structural model of TGR active sites that illustrates flow of reducing equivalents in the enzyme. Active site cysteines and selenocysteine in TGR are highlighted in this model of TGR structure that was developed as described in Materials and Methods. Cysteines are shown in yellow and selenocysteine in red. From left to right (corresponding to the flow of reducing equivalents in the enzyme): a pair of cysteines in the N-terminal thiol/disulfide site, C-terminal selenenylsulfide site, and the active site cysteine of the Grx domain. This model is not sufficient to predict identity of the residue in the selenenylsulfide motif that interacts with other redox groups in the enzyme.

Overall, these data revealed that TGR is regulated by both selenium availability and Sec tRNA status. However, this regulation was more pronounced in liver, whereas in testes, the expression level was only slightly affected. Moreover, regulation of TGR was similar to that of selenoproteins considered to have high priority for selenium supply, such as TR1 and GPx4, and was in contrast to the expression of several stress-related selenoproteins, such as GPx1, which are highly regulated by selenium and Sec tRNA status.

DISCUSSION

Thioredoxin reductases are key enzymes that control the redox state of thioredoxins (*2*). Mammalian TRs are selenoproteins, and therefore, the thioredoxin system in mammals is dependent on the availability of the trace element, selenium. The catalytic mechanism of mammalian TR1 has been well-characterized. Sec has been shown to be essential for the reduction of thioredoxin (*50*, *51*) and is thought to transfer reducing equivalents from the thiol/disulfide active site of the enzyme to the active site of thioredoxin. TGR represents a new type of TR as it contains an additional Grx domain at the N-terminus. This fusion was proposed to be responsible for the multiple functions of TGR, including TR, GR, and Grx activities (*24*). In the TGR structural model, the C-terminal Sec-containing active center is located between the N-terminal active site and the active cysteine of Grx domain, suggesting the following direction of electron flow: N-terminal thiol/disulfide active site → C-terminal selenenylsulfide active site → active site cysteine of the Grx domain (Figure 8). However, neither specific functions/contributions of individual domains of TGR nor experimental details of the proposed mechanisms have previously been determined.

TGR has a close evolutionary relationship to TR1, indicating it recently branched from this enzyme. Phylo-

genetic analyses revealed that TGR and TR1 generally co-occur in mammals (although TGR could not be identified in some mammalian genomes, it is possible that this was due to incomplete genome sequences and lack of ESTs). However, TGR did not occur in fruit fly and nematode genomes. Interestingly, TGR genes were detected in various fishes, in which TR1 was absent. Thus, it is highly likely that the fish TGR functionally replaces TR1, and in addition, it has broad substrate specificity due to presence of the Grx domain. However, TR1 reemerged in frogs, and from this point in evolution, it appears that TR1 took over functions associated with thioredoxin reduction, whereas TGR has developed into a tissue-specific specialized protein.

To further characterize the catalytic mechanism and functions of TGR, we prepared recombinant TGR forms, including the wild-type Sec-containing form and various mutant forms. These recombinant TGR forms migrated at the same position (65 kDa) as wild-type TGR on SDS−PAGE gels. In addition, a minor 130 kDa band was observed in purified protein preparations, as well as in western blots, suggesting an incomplete disruption of the TGR homodimer in these conditions. Enzymatic characterization of the purified native TGR protein, various recombinant TGR forms, and individual domains directly supported the proposal that Sec was not only required for the TR activity of TGR but also crucial for the GR and Grx activities of the enzyme in the presence of NADPH. Our current view on the reaction mechanism of TGR is shown in Figure 9. In this model, the electron transfer from the TR portion of TGR to Trx is consistent with the well-studied reaction catalyzed by TR1 (*52*, *53*). A separate path illustrates how the TR portion of the enzyme transfers electrons to the Grx domain. Thus, we established that the TR module of TGR is a universal electron transport system for all activities of TGR, suggesting the essential role of Sec in this protein.

In addition to the studies on the reaction mechanism of TGR, we assessed regulation of its expression by dietary selenium and a key regulatory mutation in the Sec tRNA gene. The data suggested that TGR has high priority for selenium supply and that its expression is dependent on selenium availability and perturbations in Sec tRNA status. Importantly, we found that the mutant form of Sec tRNA is capable of inserting Sec into TGR in both liver and testes. TGR was highly expressed in testes, whereas affinity chromatography had to be used in order to detect the protein in liver samples. Selenium has been shown to be involved in male reproduction (*54*, *55*), and the high levels of TGR in testes may be an indication that TGR, as previously shown for another selenoprotein, GPx4 (*54*), plays an important role in male reproduction.

Our study has addressed important questions related to the reaction mechanism and regulation of TGR in mammals. These findings have shown how various redox centers in TGR interact with each other and established a key role of Sec in TGR function. Furthermore, they are consistent with our recent study that suggested a role of this protein in formation and isomerization of disulfide bonds in spermatids (*30*). The Grx domain of TGR can catalyze disulfide bond isomerization reaction but requires access to reducing equivalents if nonproductive mixed disulfides are formed. From the present study, it is clear that Sec mediates the delivery of reducing equivalents from NADPH via the N-terminal disulfide active site to the Grx domain. As such, the function of TGR is expected to be dependent on the selenium status and on regulatory mechanisms associated with selenoprotein synthesis.

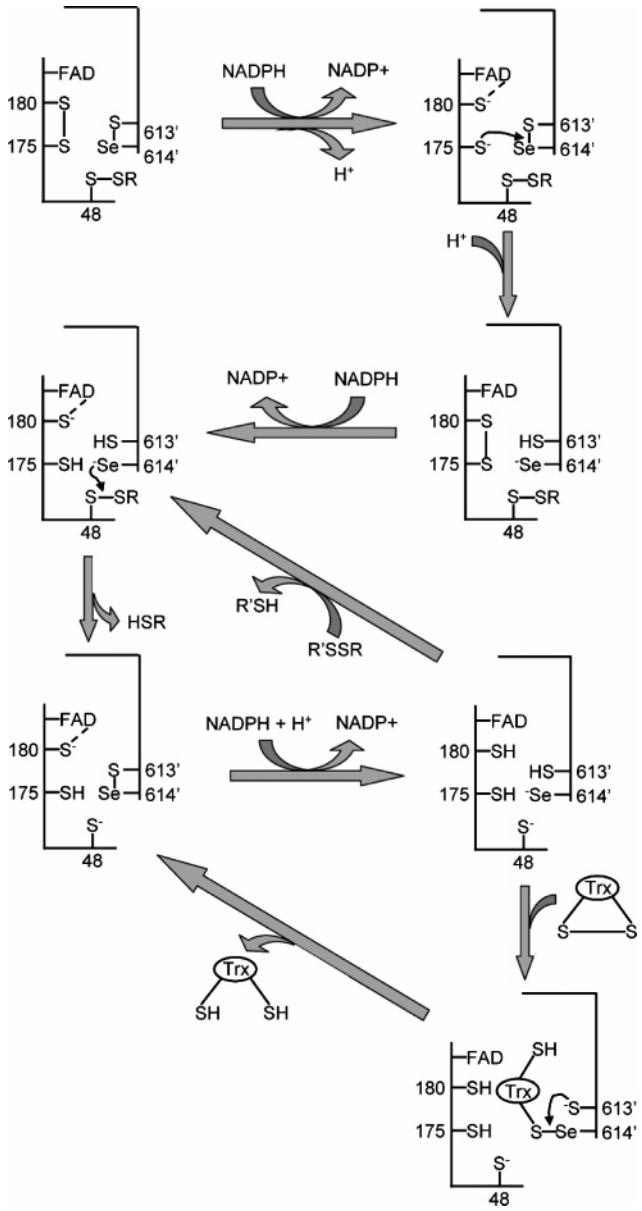

FIGURE 9: Proposed reaction mechanism of TGR. During the reaction, reducing equivalents are transferred from NADPH through FAD to the N-terminal active center ($C^{175}xxxxC^{180}$), further to the C-terminal selenenylsulfide active site ($C^{613}U^{614}$) of the other subunit in the homodimer, and finally to C^{48} of the Grx domain of the first subunit. The fully reduced enzyme can reduce either oxidized thioredoxin (Trx) using the C-terminal active site ($C^{613}U^{614}$) or other disulfide-containing substrates (RSSR′) through C^{48} of the Grx domain. R and R′ may be glutathione or other cysteine-containing substrates.

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SUPPORTING INFORMATION AVAILABLE

Expression and purification of recombinant TGR and the Grx domain. This material is available free of charge via the Internet at http://pubs.acs.org.

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