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Compensating for the Absence of Selenocysteine in High-Molecular Weight Thioredoxin Reductases: The Electrophilic Activation Hypothesis

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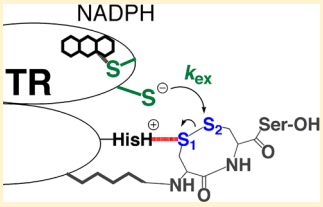
Supporting Information

ABSTRACT: Mammalian thioredoxin reductase (TR) is a pyridine disulfide oxidoreductase that uses the rare amino acid selenocysteine (Sec) in place of the more commonly used amino acid cysteine (Cys). Selenium is a Janus-faced element because it is both highly nucleophilic and highly electrophilic. Cys orthologs of Sec-containing enzymes may compensate for the absence of a Sec residue by making the active site Cys residue more (i) nucleophilic, (ii) electrophilic, or (iii) reactive by increasing both *S*-nucleophilicity and *S*-electrophilicity. It has already been shown that the Cys ortholog TR from *Drosophila melanogaster* (DmTR) has increased *S*-nucleophilicity [Gromer, S., Johansson, L., Bauer, H., Arscott, L. D., Rauch, S., Ballou, D. P., Williams, C. H., Jr., Schrimer, R. H., and Arnér, E. S (2003) Active sites of thioredoxin reductases: Why selenoproteins? *Proc. Natl. Acad. Sci. U.S.A. 100*, 12618−12623]. Here we present evidence that DmTR also enhances the electrophilicity of Cys490 through the use of an "electrophilic activation" mechanism. This mechanism is proposed to work by polarizing the disulfide bond that occurs between Cys489 and Cys490 in the C-terminal redox center by the placement of a positive charge near Cys489. This polarization renders the sulfur atom of Cys490 electron deficient and enhances the rate of thiol/disulfide exchange that occurs between the N- and C-terminal redox centers. Our hypothesis was developed by using a strategy of homocysteine (hCys) for Cys substitution in the Cys-Cys redox dyad of DmTR to differentiate the function of each Cys residue. The results show that hCys could substitute for Cys490 with little loss of thioredoxin reductase activity, but that substitution of hCys for Cys489 resulted in a 238-fold reduction in activity. We hypothesize that replacement of Cys489 with hCys destroys an interaction between the sulfur atom of Cys489 and His464 crucial for the proposed electrophilic activation mechanism. This electrophilic activation serves as a compensatory mechanism in the absence of the more electrophilic Sec residue. We present an argument for the importance of *S*-electrophilicity in Cys orthologs of selenoenzymes.



Selenium is a trace element that is used in biology in at least two ways. First, it is found in enzymes and proteins as part of the "21st" amino acid selenocysteine (Sec, U), replacing the sulfur atom of cysteine (Cys).[1,2] Second, it is found in tRNA as 2-selenouridine.[3] A key question for us and others is "Why has Nature chosen selenium?". Sulfur and selenium share many physicochemical properties because they are highly related elements as part of the chalcogen family.[4] Presumably, selenium has been selected to replace sulfur in some rare instances because it can perform chemistry that sulfur cannot.[5]

We have studied this question by dissecting the enzymatic reaction mechanism of mammalian thioredoxin reductase (TR) through a series of papers.[6−11] Others have also examined this question in studies of the reaction mechanism of TR and other selenoenzymes.[12−16] Selenium participates in the reaction mechanism of TR by two principal means as shown in Figure 1: (1) as the donor of electrons to the substrate, the disulfide bond of thioredoxin (Trx), and (2) by acting as the acceptor of electrons in the thiol/disulfide exchange step that occurs between the N- and C-terminal redox centers [recently shown by us (DOI 10.1021/bi400658g)]. Most selenoenzymes characterized to date are oxidoreductases that make use of thiol/disulfide exchange reactions of the type shown in Figure 1 in which selenium has replaced sulfur.

Thiol/disulfide exchange reactions are accelerated when (i) the strength of the attacking thiol nucleophile is increased, (ii) the ability of the sulfur atom being attacked to accept electrons (electrophilicity) is enhanced, and (iii) the stability of the leaving group is increased. Substituting selenium for sulfur in a thiol/disulfide exchange reaction will increase the rate of the reaction because of one or a combination of all three of these factors.[17] The use of selenium as a superior electron donor (superior nucleophile) relative to sulfur is the most commonly held belief about why Sec is used to replace Cys in enzymes.[18]

Cys-TR orthologs, such as the Cys-containing TR from *Drosophila melanogaster* (DmTR), must compensate for the

Received: June 7, 2013
Revised: August 13, 2013
Published: January 15, 2014

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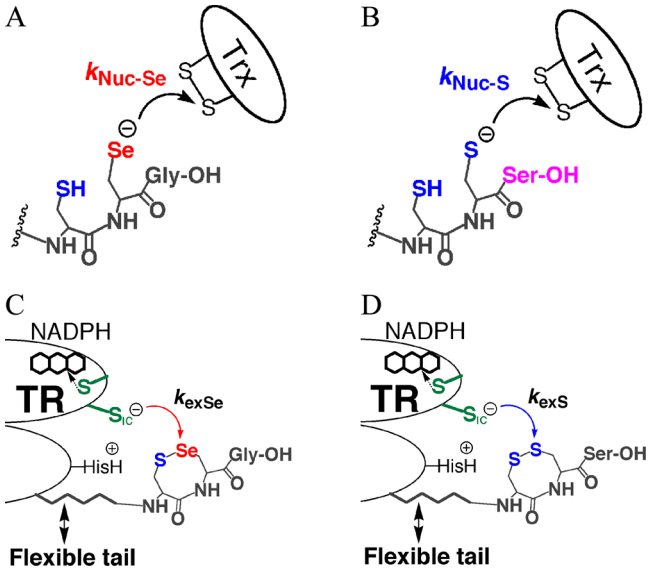


Figure 1. Key thiol/disulfide exchange reactions in the mechanism of mammalian Sec-containing TR and Cys ortholog TR from *Drosophila melanogaster*. (A) The penultimate Sec residue acts as the donor of electrons to the substrate Trx through an initial nucleophilic attack step governed by rate constant k_{Nuc-Se}. (B) The penultimate Cys residue of DmTR should act in an analogous fashion to attack the disulfide bond of the substrate with rate constant k_{Nuc-S}. To increase the nucleophilicity of the attacking thiolate, it has been proposed that flanking serine residues (magenta) help to stabilize the negative charge through hydrogen bonding.[13] These flanking Ser residues of DmTR contribute a factor of ~8 to the catalytic rate constant, k_{cat}.[13] (C) After reduction of the substrate, the vicinal selenosulfide ring of the mammalian enzyme must be reduced in the exchange step by the N-terminal redox center. The N-terminal redox center consists of an interchange Cys residue (Cys_{IC}), and a charge-transfer Cys residue (Cys_{CT}) that is involved in charge-transfer complexation with the flavin coenzyme. The selenium atom acts as the electrophile in this step (DOI 10.1021/bi400658g). (D) Analogous exchange step in DmTR. Our hypothesis is that in order for k_{exS} to approach k_{exSe}, the electrophilicity of the attacked sulfur atom must be increased.

absence of Sec by increasing the reactivity of Cys in thiol/disulfide exchange reactions by one or more of the aforementioned factors. DmTR contains a C-terminal Ser-Cys-Cys-Ser redox motif in place of a Gly-Cys-Sec-Gly motif found in the mammalian enzyme. It has been shown that the flanking serine (Ser) residues of the Cys-Cys dyad (especially the final Ser residue) help to increase the nucleophilicity of the attacking thiol by helping to stabilize the thiolate form.[13] This concept is further explained in Figure 1.

It should be expected that DmTR uses other factors mentioned above to increase the rates of the several thiol/disulfide exchange reactions that the enzyme uses during the catalytic cycle so that it would be as effective as the mammalian enzyme as a catalyst. A key thiol/disulfide exchange reaction examined in this study is the exchange step that occurs between the N-terminal redox center of the enzyme and the oxidized Cys-Cys dyad of the C-terminal redox center shown in Figure 1D (termed throughout this work simply the "exchange step").

In the analogous exchange step in the mammalian enzyme (Figure 1C), Brandt and Wessjohann proposed that selenium is used as the leaving group in this exchange step and that formation of the resulting selenolate was stabilized by the presence of a Glu-His-Sec catalytic triad.[19,20] In this model, the stabilized selenolate helps to drive the equilibrium between the oxidized N-terminal redox center and the reduced C-terminal redox center (containing the selenolate) forward, so that the selenolate could then attack the disulfide bond of Trx.[19] Replacement of Sec with Cys would no longer favor the reduction of the C-terminal redox center in this model, explaining the loss of activity in the Cys mutant.[19,21] We also proposed that selenium was used as the leaving group due to selenolate formation in this critical exchange step and that loss of leaving group ability in the Sec → Cys mutant explained the large decrease in activity.[7,9]

We recently presented evidence that selenium is not the leaving group in the exchange reaction shown in Figure 1C but is in fact the electrophile being attacked (DOI 10.1021/bi400658g). Selenium is a superior electrophile in comparison to sulfur in thiol/disulfide exchange reactions of the type shown in Figure 1C.[22−24] This should mean that DmTR would need to increase the electrophilicity of sulfur in the corresponding thiol/disulfide exchange reaction shown in Figure 1D, so that this exchange reaction does not become slow in comparison to the same reaction in the mammalian enzyme.

In this paper, we propose that the electrophilicity of the attacked sulfur atom (also termed the center sulfur atom or S_c in the literature) is increased by polarization of the disulfide bond that forms between adjacent Cys residues of the C-terminal Ser-Cys-Cys-Ser redox motif[a] as shown in Figure 2A. We term this idea the "electrophilic activation" hypothesis to explain how sulfur can become as electrophilic as selenium in analogous thiol/disulfide exchange reactions. Two similar but alternative models to electrophilic activation for increasing the rate of thiol/disulfide exchange between the N- and C-terminal redox centers of DmTR are presented in panels B and C of Figure 2.

To explain our hypothesis, we will revisit and reinterpret our data on the exchange step (Figure 1C, D). This reinterpretation will be strengthened through the use of homocysteine (hCys) replacement of each of the vicinal Cys residues of the Ser-Cys_1-Cys_2-Ser tetrapeptide to show how the geometry of the eight-membered ring of the oxidized Cys_1-Cys_2 dyad is critical to rate acceleration of the exchange step shown in Figure 1D. Our experiments with hCys will also show that it is the penultimate Cys_2 residue that acts as both the acceptor of electrons from the N-terminal redox center and the donor of electrons to the substrate. This is in complete agreement with our recent results with the mammalian mitochondrial enzyme for which we showed that the penultimate Sec residue of the Gly-Cys_1-Sec_2-Gly redox motif also acts an acceptor and donor of electrons in an analogous fashion (DOI 10.1021/bi400658g), and as originally proposed by modeling studies of the oxidized Gly-Cys_1-Sec_2-Gly redox motif in the crystal structures of mouse mitochondrial enzyme[12] and the rat cytosolic enzyme.[26]

MATERIALS AND METHODS

Materials. NADPH was purchased from AppliChem (Darmstadt, Germany). DEAE resin was obtained from Sigma-Aldrich (St. Louis, MO). Phenyl sepharose resin was from Pharmacia-Amersham Biosciences (Uppsala, Sweden). Microcon Ultracel YM-50 ultrafiltration devices by Millipore (Billerica, MA) were used for concentrating enzyme samples. 2-Chlorotritylchloride resin was from Novabiochem (San Diego, CA). Fmoc amino acids were from Synbiosci Corp. (Livermore, CA), except for Fmoc-homocysteine, which was from Bachem (King of Prussia, PA). CLEAR-OX resin was from Peptides International (Louisville, KY). Primers for DmTR mutants

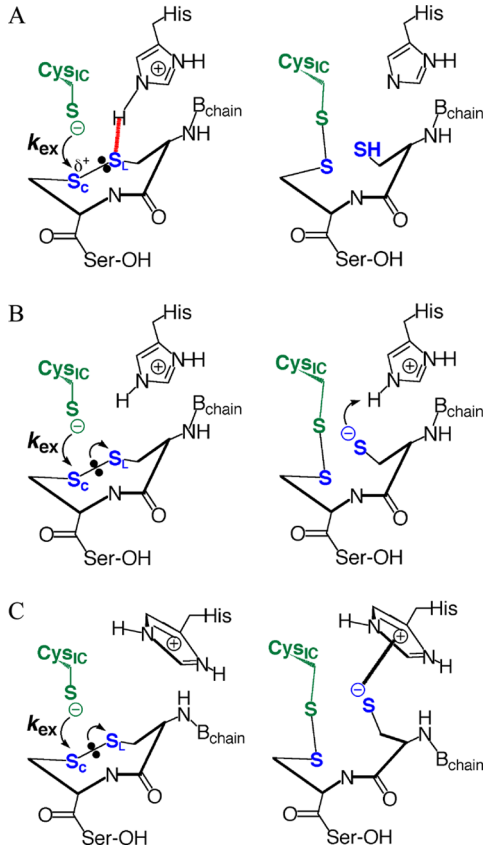


Figure 2. Electrophilic activation and leaving group stabilization models for the exchange step. (A) We propose that S_c becomes electron deficient through polarization of the shared electrons in the disulfide bond. These electrons would be drawn near S_L because of the proximity of the positively charged His464 that acts as the general acid/general base catalyst in the DmTR reaction mechanism.[25] In the ground state, lone pairs of electrons of S_L could hydrogen bond with the imidazolium group of His464. In the transition state, this hydrogen bond could strengthen, increasing the polarization of the disulfide bond, resulting in its labilization. The result would be an electron deficient S_c, which in turn would lower the barrier for the transfer of electrons from Cys_{IC} to S_c. (B) An alternative model to electrophilic activation is leaving group stabilization by use of a general acid to protonate the leaving group. In this model, the electrons in the disulfide bond between S_c and S_L are evenly shared. When Cys_{IC} attacks S_c, the electrons in the disulfide bond would move toward S_L and then attack the proton from the nearby imidazolium cation. (C) A second alternative to the one presented in panel B is stabilization of the leaving group by ion pair formation.

were from IDT (Coralville, IA), and plasmid pTYB3 and restriction enzymes were from New England Biolabs (Ipswich, MA). The DmTR-SG construct was synthesized by Retrogen, Inc. (San Diego, CA). Enzyme kinetic assays were performed on a Cary50 UV−vis spectrophotometer (Walnut Creek, CA), and all enzymatic assays were conducted at room temperature unless otherwise noted. All other chemicals were from Fisher Scientific (Fair Lawn, NJ) or Acros Organics (Morris Plains, NJ).

Peptide Synthesis. All peptides in this study were synthesized on 2-chlorotritylchloride resin using standard Fmoc chemistry as previously detailed.[27,28] Peptides were cleaved from the resin using trifluoroacetic acid containing triisopropylsilane and water in a 96:2:2 ratio. The cleavage volume was reduced by evaporation under a stream of N_2, and the peptides were then precipitated in ice-cold diethyl ether. Once dry, the peptides were redissolved in 5−10% acetonitrile in water, lyophilized, and analyzed by both MALDI-TOF mass spectrometry and analytical HPLC.

8-mer peptides, H-PTPASCCS-OH (**I**), H-PTPASC(hC)S-OH (**IV**), and H-PTPAS(hC)CS-OH (**V**) were subsequently oxidized to their cyclic forms by using CLEAR-OX resin. Briefly, a 3-fold excess of resin (vs a molar amount of peptide) was swelled in dichloromethane (DCM) for 40 min, after which the resin was washed with (three times each) DCM, dimethylformamide, methanol, deionized water, and a degassed 1:1 100 mM ammonium bicarbonate/acetonitrile mixture. Peptides were then dissolved in the degassed ammonium bicarbonate/acetonitrile mixture and added to the resin. After gentle agitation for 90 min, the peptide solution was filtered away from the resin and lyophilized. Oxidized peptides were purified by HPLC and lyophilized to obtain the crystalline, oxidized 8-mers.

Production of Semisynthetic Mutants of DmTR. A brief description of the nomenclature used in Tables 2−6 (and throughout) is warranted here. The C-terminal redox tetrapeptide of DmTR contains the amino acids Ser-Cys-Cys-Ser-OH. The wild-type (WT) enzyme is abbreviated as DmTR or DmTR-SCCS. The abbreviation DmTR-SCCS refers to the main body of the enzyme plus the C-terminal tetrapeptide, especially when we are referring to amino acid changes made to the C-terminal redox center throughout this work. Thus, in Tables 2−4, mutants are abbreviated as DmTR-aa1aa2aa3aa4 (using one-letter codes for the amino acids). The C-terminal redox center of the mouse mitochondrial TR contains the amino acids Gly-Cys-Sec-Gly-OH. Therefore, it is abbreviated as either mTR3 or mTR3-GCUG.[b] In Tables 5 and 6, the mutants of mTR3 are abbreviated as mTR3-aa1aa2aa3aa4, using the same manner of abbreviation that is used for mutants of DmTR. The production of the full-length, semisynthetic WT enzyme and mutant enzymes by intein-mediated peptide ligation (IPL) has been previously described for mTR3 (enzymes **13**−**16**) in refs 6−11 and DOI 10.1021/bi400658g and for DmTR (enzymes **1**, **2**, and **11**) in ref 8 as listed in Tables 2 and 3. For the production of enzymes **3**−**5**, **10**, and **12**, a DmTRΔ3−intein fusion protein (missing the final three amino acids of DmTR) was cleaved in the presence of peptide and 50 mM *N*-methylmercaptoacetamide (NMA) in cleavage buffer [50 mM MOPS buffer (pH 8.0) with 150 mM NaCl]. Cleavage of the DmTRΔ3−intein fusion protein with either L-cysteine (25 mM) or L-homocysteine (25 mM) dissolved in cleavage buffer but in the absence of NMA yielded enzymes **6** and **7**. The C-terminus of the DmTRΔ3−intein fusion protein is tagged with a chitin binding domain (CBD) to allow for affinity purification and cleavage on chitin agarose beads after recombinant expression in *Escherichia coli* cells.[29] After cleavage, enzymes were purified using hydrophobic and anion exchange chromatography as described previously.[6−8]

For the production of enzymes **8** and **9**, the DmTRΔ3 fusion protein construct was modified to include an additional codon (GGT, Gly) between DmTR and the intein/CBD to give a mutant where Cys_1 of the Ser-Cys_1-Cys_2-Ser tetrapeptide was mutated to Gly, resulting in a new DmTRΔ2-intein/CBD fusion construct. This construct was cleaved from the intein by adding either 25 mM L-homocysteine in cleavage buffer or 25 mM L-cysteine in cleavage buffer to the chitin agarose resin. The resin was incubated overnight at 4 °C with gentle agitation to produce enzymes **8** and **9**, respectively. The enzymes were

dx.doi.org/10.1021/bi4007258 | *Biochemistry* 2014, 53, 664−674

then purified as described above. After being purified, enzymes were concentrated using ultrafiltration. Homodimeric enzyme concentrations were determined using an extinction coefficient of 22.6 mM^{-1} cm^{-1} for flavin adenine dinucleotide (FAD).

Enzymatic Assays of TR. The assays for Trx and selenocystine as substrates of TR have been previously described.[6,7,10,30,31] Assay conditions for Trx with WT and mutant TRs consisted of 50 mM potassium phosphate buffer (pH 7.0) with 1 mM EDTA, 150 μM NADPH, and 170 μM bovine pancreatic insulin with varying concentrations of *E. coli* Trx. Assays with selenocystine as the substrate contained 500 mM potassium phosphate (pH 7.0), 10 mM EDTA, 200 μM NADPH, and varying concentrations of selenocystine. Both assays monitor the consumption of NADPH by following the loss of absorbance at 340 nm (A_{340}).

Peptide Complementation Assays. To analyze the reduction of the TR C-terminus by its N-terminal disulfide, truncated DmTR without its final eight amino acids (DmTRΔ8, enzyme **2**) was assayed with oxidized 8-mer peptides corresponding to the eight deleted C-terminal residues of enzymes **1**, **3**, and **4**. Assays contained 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 150 μM NADPH, and varying concentrations of peptide. Assays with H-PTPASCCS-OH_{ox} (**I**) contained 25 nM DmTRΔ8, and assays with both H-PTPASC(hC)S-OH_{ox} (**IV**) and H-PTPAS(hC)CS-OH_{ox} (**V**) contained 610 nM DmTRΔ8. The reduction of each peptide was monitored by the decrease in A_{340}.

Enzyme Crystallization. A crystal of enzyme **8** was grown by the vapor diffusion hanging drop method. The crystal was grown by adding 3 mL of 36 mg/mL protein in 10 mM magnesium sulfate, 50 mM ammonium citrate, 50 mM Tris (pH 8.5), and 10% glycerol mixed with 1.5 mL of a precipitant solution containing 1.6 M magnesium sulfate, 100 mM MES (pH 6.5), and 0.5 mL of 10 mM $NADP^+$ and incubated at 4 °C. Orange crystals grew in 10 days.

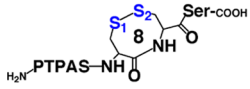
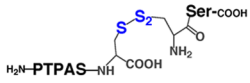
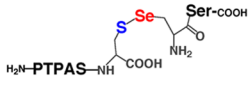
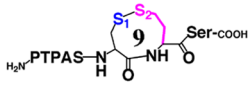
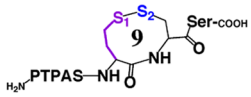
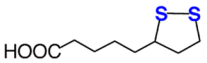
RESULTS AND DISCUSSION

The Electrophilic Activation Hypothesis Explained. Part 1: Isolation of the Exchange Reaction from the Rest of the Catalytic Mechanism. It is already recognized that Cys orthologs of Sec enzymes increase *S*-nucleophilicity to increase the rate of thiol/disulfide exchange reactions[13] to chemically behave more like the strong nucleophile selenium. However, selenium is also more electrophilic than sulfur,[22−24] so Cys orthologs of Sec enzymes should also increase *S*-electrophilicity to mimic the chemistry of selenium. The electrophilic activation hypothesis is simply that Cys orthologs of Sec enzymes must (or may) increase *S*-electrophilicity to match *Se*-electrophilicity so that the corresponding thiol/disulfide exchange reactions that occur in the Cys ortholog will be as fast as thiol/selenosulfide exchange reactions in Sec enzymes (Figure 1C, D).

Our hypothesis is that DmTR increases *S*-electrophilicity by *polarizing* the disulfide bond of the C-terminal redox center by placement of a positive charge on one end of the disulfide to mimic the polarization that occurs in the selenosulfide bond of the mammalian enzyme (Figure 2). In other words, the disulfide bond is "electrophilically activated" for attack. This means that DmTR is heavily dependent on the geometry of the active site, and especially dependent on the ring geometry of the eight-membered disulfide ring that results from oxidation of the vicinal Cys_1-Cys_2 dyad of the C-terminal redox center. Vicinal disulfide ring structures occur at a low frequency in the Protein Data Bank, perhaps because of the strain present in such a small ring.[32] Relief of strain energy could contribute to increasing the rate of thiol/disulfide exchange in DmTR. This should be less important in the mammalian enzyme because the selenium atom is larger than the sulfur atom and the corresponding Se−S and C−Se bonds are longer than the S−S and C−S bonds in DmTR.

Evidence that the eight-membered disulfide ring of DmTR is an *especially* reactive disulfide (in the context of the active site microenvironment) comes from the data in Table 1. Here, and

Table 1. Disulfide Reductase Activity of DmTRΔ8 with Peptide Substrates with Varying Ring Sizes

Peptide Number	Peptide Structure	Ring size	Activity at 5 mM peptide (mol NADPH/min/mol enzyme)	Relative Activity
I		8	230[a]	1150
II		Linear	0.2[a]	1
III		Linear	145[a]	725
IV		9	2.3	11.5
V		9	4.3	21.5
Lipoic acid		5	21[b]	105

[a]Reported in ref 9. [b]Reported in ref 10.

previously,[9,10] we used a truncated DmTR missing the C-terminal tail (DmTRΔ8) to measure the rate of reduction of various disulfide substrates as illustrated in Figure 3. These substrates include a peptide that is identical to the missing C-terminal disulfide (peptide **I**), various permutations of this peptide to test features of ring geometry (peptides **II**−**V**), and lipoic acid. While the native, eight-membered ring disulfide (peptide **I**) is somewhat strained, it is not as strained as the disulfide bond of lipoic acid,[33] yet it is turned over 11-fold faster. Further evidence that the native peptide is activated for reduction in the enzyme active site comes from comparing the turnover rate of **I** with that of **II**. Peptide **II** has an amino acid sequence identical to that of **I**, but the ring has been disrupted by removal of the amide bond between the adjacent Cys residues. This comparison demonstrates the importance of the geometry of the eight-membered ring to the "activation" of the disulfide. However, the linear form of the peptide becomes "activated" by substituting Se for S (peptide **III**). Comparison of **I** with **III** clearly shows that there is not a requirement for specific substrate geometry as long as Se is present in the scissile bond.[c]

Here we show that even very subtle changes to the ring of the native peptide result in "deactivation" of the disulfide. Peptides **IV** and **V** have incorporated hCys in place of Cys at either position of the Cys_1-Cys_2 dyad[a] to help differentiate their

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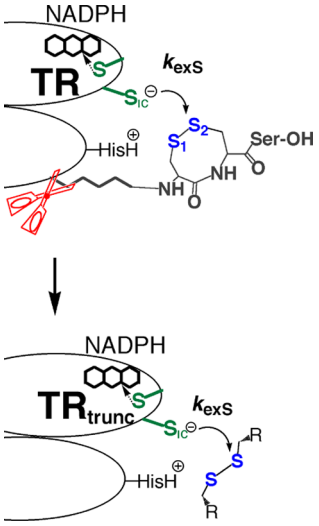

Figure 3. Strategy for isolating the exchange step in the reaction mechanism. Here, and previously,[9,10] we make use of a truncated enzyme missing the C-terminal redox center (constructed by using standard recombinant DNA methodologies) in conjunction with disulfide-containing substrates to gain a better understanding of factors that effect the thiol/disulfide exchange reaction that occurs between the N- and C-terminal redox centers. This strategy allows us to use linear disulfide substrates, cyclic disulfide substrates, or substrates in which Se replaces S to study the effects on this exchange step.

individual functions. The side chain of hCys is one methylene unit longer (~3 Å) than Cys, and an oxidized Cys-hCys dyad results in the formation of a nine-membered ring instead of an eight-membered ring from an oxidized Cys-Cys dyad. As shown by the data in Table 1, peptides with either a Cys_1-$hCys_2$ dyad or a $hCys_1$-Cys_2 dyad are reduced 100-fold slower (peptide **IV**) or 50-fold slower (peptide **V**) by the truncated enzyme than the native peptide (**I**). This shows that the precise positioning of the two sulfur atoms of the substrate disulfide bond is critical for its activation and reduction in the active site of DmTR. We again emphasize that precise positioning of substrate atoms in the active site is NOT required if the substrate disulfide bond is replaced with a selenosulfide bond, as in the case of peptide **III**. We contend that this is due to polarization of the scissile bond conferred by the presence of the selenium atom. In other words, selenium is highly electrophilic and can easily accept electrons from Cys_{IC} in the exchange step, *irrespective* of the geometric arrangement of the substrate atoms. This concept was further tested by experiments described in the next section.

The Electrophilic Activation Hypothesis Explained. Part 2: Alteration of Ring Geometry in DmTR by Backbone Lengthening and Homocysteine Substitution. Our hypothesis was tested by constructing mutants of DmTR using both conventional methodologies and protein semisynthesis, so that the C-terminal disulfide ring was altered in two different ways as illustrated in Figure 4. The previously described experimental design in Figure 3 is a system in which we add a peptide as a substrate for the truncated enzyme to measure the rate of thiol/disulfide exchange between the two enzyme redox centers. The rate of thiol/disulfide exchange was slow when the peptide substrate contained a nine-membered disulfide ring due to hCys substitution. Construction of a full-length enzyme allowed us to determine if the overall rate of Trx reduction was similarly slow when hCys substituted for Cys (enzymes **3**–**5** in Table 2).

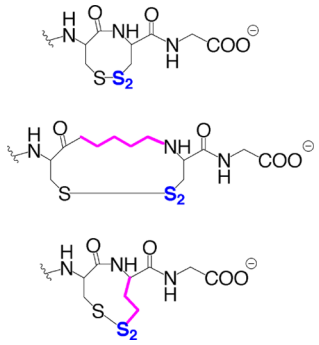

Figure 4. Methods for altering the ring geometry of the C-terminal vicinal disulfide of DmTR used in this study. The top panel shows the situation in the WT enzyme with a vicinal disulfide forming an eight-membered ring. The geometry of the disulfide ring can be altered by inserting amino acids between Cys_1 and Cys_2 as shown in the middle panel. Each addition of an amino acid increases the backbone length by three atoms (magenta). The other method for altering the ring geometry maintains the amide bond connectivity but increases the length of the side chain through the use of hCys substitition (magenta in the bottom panel).

Table 2. Trx-Reductase Activity of WT DmTR and Homocysteine-Containing Mutants[a]

enzyme	enzyme number	k_{cat} (min^{-1})	K_m (μM)	activity at 90 μM
DmTR-SCCS[b]	**1**	300 ± 7.4	173 ± 8.1	95
DmTRΔ8[c]	**2**	ND[d]	ND[d]	ND[d]
DmTR-SC**h**CS	**3**	103 ± 10	101 ± 20	48
DmTR-S**h**CCS	**4**	NA[e]	NA[e]	0.4
DmTR-S**h**C**h**CS	**5**	35 ± 2.2	143 ± 17	14
DmTR-S**h**C	**6**	ND[d]	ND[d]	ND[d]
DmTR-SC	**7**	ND[d]	ND[d]	ND[d]
DmTR-SG**h**C	**8**	ND[d]	ND[d]	ND[d]
DmTR-SGC	**9**	ND[d]	ND[d]	ND[d]
DmTR-SG**h**CS	**10**	ND[d]	ND[d]	ND[d]

[a]Please see Materials and Methods for a description of abbreviations of the mutant enzymes. [b]Reported in ref 8. [c]Reported in ref 9. [d]Not detectable. [e]Not applicable.

Our expectation was that there would be little or no Trx-reductase activity in the case of the hCys-containing full-length enzymes **3**–**5**. This was true for enzyme **4** in which hCys replaced Cys_1. Unexpectedly, however, we found that enzyme **3** in which hCys replaced Cys_2 had Trx-reductase activity very similar to that of the WT enzyme (compare enzymes **3** and **1** in Table 2). This is despite the fact that the rate of thiol/disulfide exchange between the N-terminal redox center of the enzyme and the nine-membered ring disulfide substrate of peptide **IV** was *very slow* (Table 1). Enzyme **3** also had selenocystine-reductase activity very similar to that of the WT enzyme as shown by the data in Table 3. This experiment differentiates the roles of the two Cys residues of the dyad and supports our electrophilic activation hypothesis as outlined in Figure 2.

When Cys_1 is replaced with hCys, there is little Trx-reductase activity because the enzyme relies upon Cys_1 to resolve the mixed disulfide formed between DmTR and Trx, resulting in the formation of the eight-membered ring. As the data in Table 1 demonstrate, the eight-membered disulfide ring is activated for thiol/disulfide exchange. The low Trx-reductase activity that results when hCys replaces Cys_1 can be explained in one of two ways. First, the longer side chain of hCys compared to that of

dx.doi.org/10.1021/bi4007258 | *Biochemistry* 2014, 53, 664–674

Table 3. Selenocystine Reductase Activities of WT DmTR and Mutant Enzymes[a]

enzyme	enzyme number	k_{cat} (min^{-1})	K_m (μM)	activity at 91 μM
DmTR-SCCS	**1**	1112 ± 172	1090 ± 223	110[b]
DmTRΔ8	**2**	NA[c]	NA[c]	3.3
DmTR-SC**h**CS	**3**	1213 ± 233	1536 ± 363	68
DmTR-S**h**CCS	**4**	NA[c]	NA[c]	41
DmTR-S**h**C**h**CS	**5**	520 ± 55	954 ± 137	41.6
DmTR-S**h**C	**6**	NA[c]	NA[c]	38.6
DmTR-SC	**7**	NA[c]	NA[c]	27.5
DmTR-SG**h**C	**8**	NA[c]	NA[c]	13.9
DmTR-SGC	**9**	NA[c]	NA[c]	25
DmTR-SG**h**CS	**10**	NA[c]	NA[c]	25.7
DmTR-SCAACS	**11**	113.0 ± 8.64	291.3 ± 45.0	27.9
DmTR-SCAAUS	**12**	868.2 ± 51	87.53 ± 15	454

[a]Please see Materials and Methods for a description of abbreviations of the mutant enzymes. [b]Reported in ref 10. [c]Not applicable.

Cys may result in steric hindrance that prevents ring formation between hCys and Cys, resulting in an inability of the mutant enzyme to resolve the mixed disulfide bond between TR and Trx. Second, the enzyme may use a pathway that results in the formation of a nine-membered disulfide ring, which our previous experiments show is unreactive toward reduction. However, when hCys replaces Cys_2, the enzyme can still reduce Trx efficiently because a ring formation pathway can be avoided. We term this ring avoidance pathway the "bypass mechanism" as further explained in Figure 5.

We would like to note that we can rule out inefficient peptide ligation as the reason for the low Trx-reductase activity of enzyme **4** because this mutant has one-third of the selenocystine-reductase activity (specific activity) of the WT enzyme as shown by the data in Table 3. If the peptide were not ligated to the enzyme, it would be expected to have the same selenocystine-reductase activity as the truncated enzyme (enzyme **2**).

While replacement of Cys_1 with hCys results in a mutant with almost no Trx-reductase activity, a double mutant in which both Cys residues of the Cys_1-Cys_2 dyad are replaced with hCys (enzyme **5**) has 10% of the Trx-reductase activity of the WT enzyme, supporting our hypothesis that the use of hCys in the second position of the dyad allows the enzyme to use a pathway for Trx reduction that is independent of ring formation.

As a test of our proposed bypass mechanism, we constructed mutant enzymes **8** and **10** in which the Cys_1 position was occupied by glycine. We predicted that these mutants would have Trx-reductase activity, similar to results with the mammalian enzyme with an analogous mutant [reported by us (DOI 10.1021/bi400658g)]. However, these mutants showed no Trx-reductase activity as summarized in Table 2. This was a disappointing result. However, an unexpected and redeeming result was the fact that both enzymes were orange in color. This contrasts with the typical yellow color of the isolated, oxidized enzyme (E_{ox}). The absorbance spectra of the WT and mutant enzymes show that there is significant charge-transfer complexation occurring in **8** as shown by the increased extinction coefficient at 540 nm (Figure 6A). Bauer and co-workers reported a near identical phenomenon for mutant C489S of DmTR (C489 is Cys_1 of the dyad).[34] The mutant constructed by Bauer showed more red color, indicating a higher concentration of charge-transfer complexation. The orange color of enzymes **8** and **10** indicates that they are in equilibrium between E_{ox} and a two-electron-reduced state that produces a charge-transfer complex as shown in Figure 6B. A dramatic demonstration of the formation of this oxidation state of the enzyme is shown in the inset of Figure 6A, which shows a picture of an orange crystal of enzyme **8**.

The kinetic and spectral data of enzymes **3**, **8**, and **10** demonstrate that atom S_2 of Cys_2 (Cys490) is the atom responsible for *accepting* electrons from the N-terminal redox center and then *donating* them to the substrate. Cys490 of DmTR occupies the same position in the C-terminal redox dyad as Sec does in the mammalian enzyme. We recently presented evidence that the Se atom of Sec both accepts and donates electrons in an identical fashion (DOI 10.1021/bi400658g). The evidence presented here that the sulfur atom of Cys490 is attacked in the thiol/disulfide exchange step that occurs between N- and C-terminal redox centers is consistent with structural observations from both the mammalian mitochondrial TR[12] and the mammalian cytosolic TR[26] in which attack at the Se atom seemed the most likely in the analogous thiol/selenosulfide exchange reaction. We emphasize that Bauer and co-workers first proposed that the sulfur atom of Cys490 (Cys_2) acted as the acceptor of electrons in the exchange step based upon spectroscopic data.[34] Our data using hCys substitution confirm this finding.

Thus, both the mammalian Sec-containing enzyme and the Cys ortholog from *D. melanogaster* use nearly identical catalytic mechanisms. This bolsters our hypothesis that Cys orthologs must increase *S*-electrophilicity to help compensate for the absence of an electrophilic selenium atom. According to our electrophilic activation hypothesis (Figure 2), a key difference between the mechanism of the Sec-TR and the Cys ortholog TR is that the Cys ortholog makes use of the enzyme microenvironment to induce polarization in the disulfide bond of the C-terminal redox center, while this type of polarization may not be needed in the mammalian enzyme because of the polarization of the selenosulfide bond induced by the selenium atom itself.

In addition to altering ring size by increasing the length of the side chain via hCys substitution, we also increased ring size by *lengthening the backbone* between Cys_1 and Cys_2 by the insertion of two alanine residues (enzyme **11** in Table 4) reported by us previously.[8] Increasing the backbone length results in a 300-fold decrease in k_{cat} compared to that of the WT enzyme. This decrease in activity can be explained in one of two ways in the mutant: (i) loss of nucleophilicity of Cys_2 or (ii) loss of electrophilic activation of the Cys_1-Ala-Ala-Cys_2 disulfide due to disruption of our proposed $HisH^+$:::S-Cys_1 interaction. Here, we revisited this experiment with the thought of rescuing the activity of the enzyme via Se substitution at the Cys_2 position (enzyme **12**) to test our hypothesis that ring geometry is nearly irrelevant to the exchange reaction if selenium is present in the C-terminus. As the data in Table 4 show, the Trx-reductase activity of enzyme **12** is "rescued", approaching the activity of the WT enzyme. The conventional thinking in the field would be to ascribe the rescue effect to the greater nucleophilicity of Se relative to that of S. However, this rationale does not match the data in Table 3, where enzyme **12** has only 8−16-fold higher activity (as measured by k_{cat} or specific activity) with selenocystine as the substrate compared to enzyme **11**. Moreover, enzyme **12** has only ∼4-fold higher specific activity with selenocystine as the substrate compared to

dx.doi.org/10.1021/bi4007258 | *Biochemistry* 2014, 53, 664−674

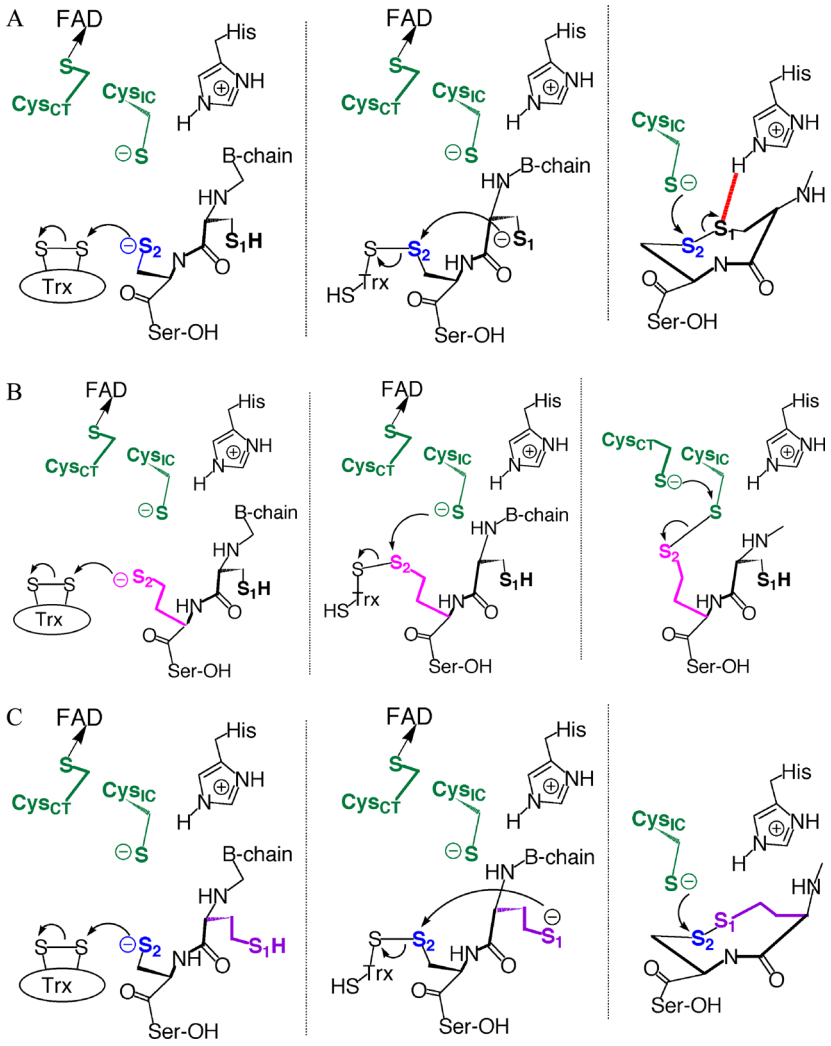

Figure 5. Partial mechanisms for WT DmTR and hCys mutants. For a description of the complete catalytic mechanism, please see ref 34. As presented here, the mechanism proceeds from left to right and begins with initial nucleophilic attack by S_2 on the disulfide bond of Trx. (A) The mechanism of WT DmTR must use a pathway that involves resolution of the mixed disulfide between the enzyme and Trx resulting in the formation of the eight-membered ring. Shown in the far right portion of panel A is our proposal for how the C-terminal disulfide is electrophilically activated for attack via the $HisH^+$:::S-Cys_1 interaction (indicated by the red hash marks). (B) When hCys replaces Cys490 (Cys_2), the activity of the mutant enzyme (enzyme **3**) is close to that of the WT enzyme even though the electrophilic activation mechanism is disrupted. The longer side chain of hCys permits direct resolution of the mixed disulfide bond between TR and Trx via attack by Cys_{IC}. We term this mechanism the bypass mechanism. (C) The electrophilic activation mechanism is disrupted when hCys replaces Cys489 (Cys_1) in enzyme **4** because the enzyme must use a ring formation pathway involving a nine-membered ring that disrupts the key $HisH^+$:::S-Cys_1 interaction proposed by us resulting in impaired catalysis.

that of the WT enzyme (enzyme **1**), or 10-fold higher as measured by k_{cat}/K_m.

While *Se*-nucleophilicity undoubtedly does make a contribution to the rescue effect, a significant part of the rescue effect would come by increasing the rate of the exchange reaction. This could occur through the use of an alternative mechanism of Trx reduction in mutant **12** as shown in Figure 7. Further support for the contribution of *Se*-electrophilicity as a primary factor in the rescue effect comes from data with the analogous mutant in the mammalian enzyme (enzyme **16** in Table 5). Enzyme **16** does have reduced Trx-reductase activity, but it is only reduced 4-fold compared to that of the WT enzyme. In contrast, lengthening the backbone of the C-terminal dyad results in a 300-fold decrease in activity in the Cys ortholog (compare enzyme **11** with enzyme **1** in Table 4). Furthermore, mutant enzyme **16** has selenocystine-reductase activity nearly identical to that of the WT enzyme (Table 6). These data suggest to us that enzyme **16** also does not use a pathway involving formation of a C-terminal ring for the reduction of substrates.

Further Evidence of the Electrophilic Activation Hypothesis: A "Seleno Effect" in DmTR Can Be Explained by an Electrophilic Activation Mechanism. It has been well-known for many decades that phosphodiesterases exhibit a "thio effect" when S for O substitution occurs in a nonbridging oxygen atom of a substrate phosphate group.[35−37] There is strong evidence that the phosphorus atom is activated for nucleophilic attack by a hydrogen bonding/electrostatic interaction of the nonbridging phosphate oxygen with a positively charged hydrogen bond donor.[38] This interaction not only stabilizes the negative charge built up in the transition state but also *polarizes* the P−O bond, thereby activating the phosphorus center for nucleophilic attack. One proposal for the way in which the phosphorus atom is activated is via the so-

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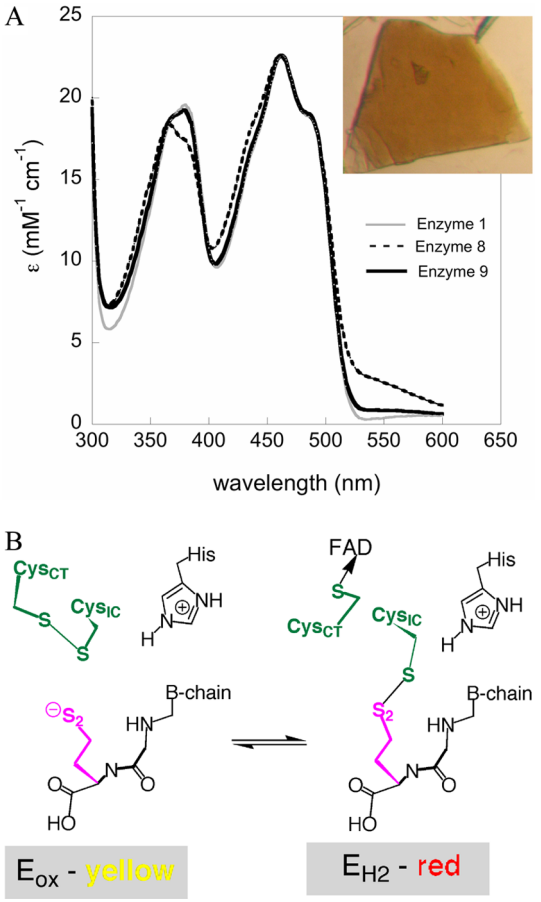

Figure 6. (A) Extinction vs wavelength plot for WT and mutant enzymes. The plot shows an increase in the extinction coefficient at 540 nm for DmTR-SG**hC** (enzyme **8**), which contains hCys in place of Cys490. This increase in the extinction coefficient is explained by back attack of the sulfhydryl of the hCys residue on the disulfide bond of the N-terminal redox center. This results in charge-transfer complexation of the flavin ring and is observed spectroscopically as shown here as well as visibly by the enzyme taking on an orange color. The top right inset shows a crystal of enzyme **8** grown by the vapor diffusion hanging drop method as described in Materials and Methods. (B) Diagram of the equilibrium hypothesized to produce the orange color of enzyme **8** in solution and in the crystal. When the enzyme is oxidized, the flavin takes on the characteristic yellow color (left). The long side chain of the homocysteine permits back attack on the N-terminal disulfide and results in a reduced flavin that is red (right). A rapid equilibrium of the two structures would produce the observed orange color of the mutant enzyme.

Table 4. Trx-Reductase Activity of WT DmTR and Backbone Mutants[a]

enzyme	enzyme number	k_{cat} (min^{-1})	K_m (μM)
DmTR-SCCS[b]	**1**	300 ± 7.4	173 ± 8.1
DmTR-SCAACS[b]	**11**	1 ± 0.2	166 ± 58
DmTR-SCAAUS	**12**	153 ± 6.8	19 ± 3.3

[a]Please see Materials and Methods for a description of abbreviations of the mutant enzymes. [b]Reported in ref 8.

called "low-barrier" hydrogen bond, which involves pK_a matching between the donor and acceptor,[39] like what *might* occur for $HisH^+$:::S-Cys_1 in DmTR (Figure 2A).

We have previously described an analogous seleno effect in DmTR[40] that supports our proposed $HisH^+$:::S-Cys_1 interaction. Our previous results show that when we used DmTRΔ8 and peptide substrates in which each Cys residue of the dyad is replaced with Sec, there is a 12-fold increase in turnover rate relative to that of peptide **I** when Cys_2 is replaced with Sec

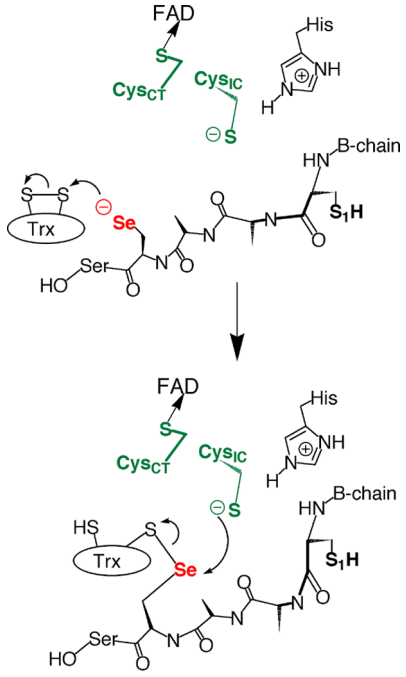

Figure 7. Proposed direct resolution mechanism of the enzyme–substrate complex by Cys_{IC} in enzymes **12** and **16**. This alternative mechanism is consistent with our proposed bypass mechanism used by enzyme **3** (Figure 5B). The Trx-reductase activity of **16** decreases 4.5-fold relative to that of the WT mammalian enzyme, while the Trx-reductase activity of **12** is decreased 2-fold relative to that of the WT DmTR enzyme. Previous results with mTR3 using a mutant containing a Gly-Gly-Sec motif in place of the Gly-Cys-Sec-Gly motif of the WT enzyme showed that the mutant could still reduce Trx (DOI 10.1021/bi400658g), and this shows proof of concept of our proposed direction resolution mechanism shown here.

Table 5. Trx-Reductase Activities of WT mTR3 and Mutant Enzymes[a]

enzyme	enzyme number	k_{cat} (min^{-1})	K_m (μM)
mTR-GCUG[b]	**13**	2220 ± 78	67.6 ± 6
mTRΔ8[c]	**14**	ND[e]	ND[e]
mTR-GCCG[b]	**15**	4 ± 0.1	49 ± 3
mTR-GCAAUG[d]	**16**	500 ± 40	35 ± 10

[a]Please see Materials and Methods for a description of abbreviations of the mutant enzymes. [b]Reported in ref 6. [c]Reported in ref 9. [d]Reported in ref 8. [e]Not detectable.

Table 6. Selenocystine Reductase Activities of WT mTR3 and Mutant Enzymes[a]

enzyme	enzyme number	k_{cat} (min^{-1})	K_m (μM)	activity at 91 μM
mTR-GCUG	**13**	1403.6 ± 128	341.2 ± 59.0	295
mTRΔ8[b]	**14**	NA[c]	NA[c]	0.6
mTR-GCCG	**15**	681.7 ± 91.5	791.3 ± 151.7	78.4
mTR-GCAAUG	**16**	1228 ± 100	205 ± 40	415.5

[a]Please see Materials and Methods for a description of abbreviations of the mutant enzymes. [b]Reported in ref 10. [c]Not applicable.

dx.doi.org/10.1021/bi4007258 | *Biochemistry* 2014, 53, 664–674

(peptide **VI**), and a 10-fold decrease in turnover rate relative to that of peptide **I** when Cys_1 is replaced with Sec (peptide **VII**). The proposed seleno effect is shown in Figure S1 of the Supporting Information. Our original explanation for this phenomenon was that the increase in activity due to Sec substitution at the Cys_2 position was caused by the superior leaving group ability of Se relative to that of S. We ascribed the decrease in activity to Sec substitution at the Cys_1 position from attack on the "incorrect atom".[9] Our electrophilic activation hypothesis offers a much better explanation of this data. If it is S_2 of Cys_2 that is attacked by Cys_{IC}, then Se substitution should cause an increase in the rate because of the superior electrophilicity of Se relative to that of S. Likewise, Se substitution for S_1 would cause a decrease in rate because Se is less basic than S and thus cannot as easily hydrogen bond or accept a proton from His464. This decrease in basicity relative to that of S would *deactivate* the Se_1-S_2 bond, resulting in a slower rate.

Does Glutathione Reductase Also Use an Electrophilic Activation Mechanism? There is a strong evolutionary relationship between glutathione reductase and TR, and the C-terminal redox center of TR can be considered a glutathione-like module that is covalently linked to the enzyme that shuttles electrons from the N-terminal redox center to the substrate.[41] This evolutionary relationship is underscored by the structural relationship between bound oxidized glutathione and a peptide vicinal disulfide bond as depicted in Figure S2 of the Supporting Information.

Blanchard and co-workers proposed that the rate-determining step in the reaction mechanism was the transfer of a proton from a catalytic histidine to the thiolate of the glutathione anion during the analogous exchange step in the TR reaction mechanism.[42] The exchange step in the glutathione reductase mechanism is identical to the exchange step between N- and C-terminal redox centers in the TR mechanism shown in panels C and D of Figure 1. For the DmTR mechanism, this would mean the transfer of a proton to Cys489 (Cys_1) from His464 as shown in Figure 2B is the rate-determining step. Our electrophilic activation idea (Figure 2A) is very similar to this proton-transfer mechanism. The difference between our hypothesis presented here and Blanchard's model is that we have considered *Se*-electrophilicity in the Sec-TR and, thus, the corresponding *S*-electrophilicity in the Cys ortholog as a way to enhance thiol/disulfide exchange reactions. The electrophilic activation model might be described as a more refined version of Blanchard's original model as can be seen from the similarities between the models presented in Figure 2.

Which Model Is Correct? Enzymologists studying thiol/disulfide exchange reactions have only considered two possibilities for increasing the rates of these reactions: increasing the strength of the thiolate nucleophile and increasing the stability of the leaving group thiolate.[43−46] With the discovery that Sec participates in analogous thiol/disulfide exchange reactions in Sec-containing enzymes, this view must be modified because of the high electrophilicity of selenium.[17] The key concept we are trying to convey here is that Cys orthologs of Sec-containing enzymes should also increase *S*-electrophilicity of the center sulfur atom to help labilize the disulfide bond to more closely match the labilization/polarization of a selenosulfide bond imparted by the presence of the selenium atom, as our data in Table 1 clearly indicate.

In this paper and previously,[7,9] we have shown the importance of the thiol/disulfide exchange reaction that occurs between the N- and C-terminal redox centers to the overall catalytic reaction mechanism. In Figure 2, we have presented three different models for how this exchange reaction could be accelerated. A key difference between the electrophilic activation model shown in panel A and the alternative models in panels B and C is that in the electrophilic activation model, the disulfide bond is labilized (i.e., activated for attack), whereas models B and C only stabilize the product of the reaction. Bond polarization as a method for labilizing bonds is a well-established principle in enzymology.[47,48]

Key to our electrophilic activation model in DmTR is polarization of the C-terminal disulfide by His464. Electron density from the C-terminal disulfide is not present in the crystal structure of DmTR,[7] but our modeling studies show that His464 can be placed close to the sulfur atom of Cys489[7] as we depict in Figure 2A, demonstrating the feasibility of our proposed mechanism. The equivalent His residue in rat cytosolic TR is His472. Modeling studies of the oxidized Cys-Sec dyad of the C-terminal redox center of this enzyme show that the selenium atom of Sec498 appears to be somewhat closer to the imidazolium cation than the sulfur atom of Cys497 (Cys_1) is to His472.[26] The mammalian enzyme may not use an electrophilic activation mechanism of the type proposed here because of the polarization of the -S−Se- bond conferred by selenium itself. A crystal structure showing the C-terminal disulfide positioned next to the N-terminal redox center in DmTR would help substantiate our interpretation of our data and the hypothesis we have presented here.

■ CONCLUSIONS

We recently presented evidence that the selenium atom from the C-terminal Cys_1-Sec_2 redox dyad is responsible for *both* accepting and donating electrons in the enzymatic reaction cycle (DOI 10.1021/bi400658g). This is because selenium is a Janus-faced element, as it is both strongly nucleophilic and strongly electrophilic. Here we have shown that the analogous sulfur atom of the C-terminal Cys_1-Cys_2 redox dyad in the Cys ortholog TR is also responsible for accepting and donating electrons in the enzymatic reaction cycle. We have presented evidence that Cys orthologs of Sec enzymes compensate for the absence of Sec by increasing *S*-electrophilicity as a way of accelerating thiol/disulfide exchange reactions. This is a concept that has not been considered heretofore.

■ ASSOCIATED CONTENT

Supporting Information

A diagram explaining the seleno effect (Figure S1) and superposition of the disulfide bond of the Cys-Cys dyad of DmTR with that of oxidized glutathione (Figure S2). 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 Grant GM094172 to R.J.H. A.P.L. was supported by National Institutes of Health Training Grant T32 HL07594 administered by Dr. Kenneth G. Mann.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Hans J. Reich (University of Wisconsin, Madison, WI) without whose counsel we would never have developed the electrophilic activation hypothesis. We also acknowledge the Vermont Cancer Center DNA Facility for sequencing services.

■ ABBREVIATIONS

A_{340}, absorbance at 340 nm; βME, β-mercaptoethanol; CBD, chitin-binding domain; Cys, cysteine; Cys_{CT}, charge-transfer cysteine; Cys_{IC}, interchange cysteine; DCM, dichloromethane; DEAE, diethylaminoethyl; DmTR, TR from *D. melanogaster*; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; Fmoc, 9-fluorenylmethoxycarbonyloxy; Glu, glutamic acid; Gly, glycine; HPLC, high-pressure liquid chromatography; His, histidine; hCys, homocysteine; IPL, intein-mediated peptide ligation; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; M_r, molecular ratio; mTR3, mouse mitochondrial thioredoxin reductase; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; NMA, *N*-methylmercaptoacetamide; Sec, selenocysteine; Ser, serine; Tris, tris(hydroxymethyl)aminomethane; Trx, thioredoxin; TR, thioredoxin reductase; tRNA, transfer ribonucleic acid; U, one-letter code for Sec; UV−vis, ultraviolet−visible; WT, wild-type.

■ ADDITIONAL NOTES

[a]DmTR uses a Ser-Cys_1-Cys_2-Ser C-terminal redox center. We denote the N-terminal Cys with the subscript "1" and the C-terminal Cys with the subscript "2". Cys_1 corresponds to Cys488, and Cys_2 corresponds to Cys489. In Figure 2, the S atom of Cys_1 is S_L while the S atom of Cys_2 is S_C.

[b]Thioredoxin reductase is abbreviated as TR. The cytosolic enzyme is abbreviated as TR1, while the mitochondrial enzyme is abbreviated as TR3. This study was performed with the mouse mitochondrial enzyme, and it is therefore abbreviated as mTR3 or mTR3-GCUG to denote the last four amino acids of the C-terminal redox center.

[c]These data have been presented previously by us, but we concluded that Se "activated" the substrate by acting as a good leaving group.[9]

■ REFERENCES

(1) Bock, A., Forchhammer, K., Heider, J., Leinfelder, W., Sawers, G., Veprek, B., and Zinoni, F. (1991) Selenocysteine: The 21st amino acid. *Mol. Microbiol. 5*, 515−520.

(2) Atkins, J. F., and Gesteland, R. F. (2000) The twenty-first amino acid. *Nature 407*, 463−465.

(3) Ching, W. M., Wittwer, A. J., Tsai, L., and Stadtman, T. C. (1984) Distribution of two selenonucleosides among the selenium-containing tRNAs from *Methanococcus vannielii*. *Proc. Natl. Acad. Sci. U.S.A. 81*, 57−60.

(4) Wessjohann, L. A., Schneider, A., Abbas, M., and Brandt, W. (2007) Selenium in chemistry and biochemistry in comparison to sulfur. *Biol. Chem. 388*, 997−1006.

(5) Ruggles, E. L., Snider, G. W., and Hondal, R. J. (2011) Chemical basis for the use of selenocysteine. In *Selenium: Its Molecular Biology and Role in Human Health* (Hatfield, D. L., Berry, M. J., and Gladyshev, V. N., Eds.) 3rd ed., pp 73−83, Springer, New York.

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

(7) Eckenroth, B. E., Rould, M. A., Hondal, R. J., and Everse, S. J. (2007) Structural and biochemical studies reveal differences in the catalytic mechanisms of mammalian and *Drosophila melanogaster* thioredoxin reductases. *Biochemistry 46*, 4694−4705.

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

(9) Lacey, B. M., Flemer, S., Jr., Eckenroth, B. E., and Hondal, R. J. (2008) Selenium in thioredoxin reductase: A mechanistic perspective. *Biochemistry 47*, 12810−12821.

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

(11) Snider, G. W., Grout, L., Ruggles, E. L., and Hondal, R. J. (2010) Methaneseleninic acid is a substrate for truncated thioredoxin reductase: Implications for the catalytic mechanism and redox signaling. *Biochemistry 49*, 10329−10338.

(12) Biterova, E. I., Turanov, A. A., Gladyshev, V. N., and Barycki, J. J. (2005) Crystal structures of oxidized and reduced mitochondrial thioredoxin reductase provide molecular details of the reaction mechanism. *Proc. Natl. Acad. Sci. U.S.A. 102*, 15018−15023.

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

(14) Johansson, L., Arscott, L. D., Ballou, D. P., Williams, C. H., Jr., and Arnér, E. S. (2006) Studies of an active site mutant of the selenoprotein thioredoxin reductase: The Ser-Cys-Cys-Ser motif of the insect orthologue is not sufficient to replace the Cys-Sec dyad in the mammalian enzyme. *Free Radical Biol. Med. 41*, 649−656.

(15) Nauser, T., Steinmann, D., and Koppenol, W. H. (2012) Why do proteins use selenocysteine instead of cysteine? *Amino Acids 42*, 39−44.

(16) Kim, H. Y., Fomenko, D. E., Yoon, Y. E., and Gladyshev, V. N. (2006) Catalytic advantages provided by selenocysteine in methionine-*S*-sulfoxide reductases. *Biochemistry 45*, 13697−13704.

(17) Hondal, R. J., Marino, S. M., and Gladyshev, V. N. (2013) Selenocysteine in thiol-disulfide-like exchange reactions. *Antioxid. Redox Signaling 18*, 1675−1689.

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

(19) Brandt, W., and Wessjohann, L. A. (2005) The functional role of selenocysteine (Sec) in the catalysis mechanism of large thioredoxin reductases: Proposition of a swapping catalytic triad including a Sec-His-Glu state. *ChemBioChem 6*, 1−9.

(20) Gromer, S., Wessjohann, L. A., Eubel, J., and Brandt, W. (2006) Mutational studies confirm the catalytic triad in the human selenoenzyme thioredoxin reductase predicted by molecular modeling. *ChemBioChem 7*, 1649−1652.

(21) Zhong, L., and Holmgren, A. (2000) Essential role of selenium in the catalytic activities of mammalian thioredoxin reductase revealed by characterization of recombinant enzymes with selenocysteine mutations. *J. Biol. Chem. 275*, 18121−18128.

(22) Bachrach, S. M., Demoin, D. W., Luk, M., and Miller, J. V., Jr. (2004) Nucleophilic attack at selenium in diselenides and

selenosulfides. A computational study. *J. Phys. Chem. A 108*, 4040−4046.
(23) Steinmann, D., Nauser, T., and Koppenol, W. H. (2010) Selenium and sulfur in exchange reactions: A comparative study. *J. Org. Chem. 75*, 6696−6699.
(24) Reich, H., Gudmundsson, B. O., Green, D. P., Bevan, M. J., and Reich, I. L. (2002) The role of ate complexes in the lithium-sulfur, lithium-selenium, and lithium-tellurium exchange reactions. *Helv. Chim. Acta 85*, 3748−3772.
(25) Huang, H. H., Arscott, L. D., Ballou, D. P., and Williams, C. H., Jr. (2008) Acid-base catalysis in the mechanism of thioredoxin reductase from *Drosophila melanogaster*. *Biochemistry 47*, 1721−1731.
(26) Cheng, Q., Sandalova, T., Lindqvist, Y., and Arner, E. S. (2009) Crystal structure and catalysis of the selenoprotein thioredoxin reductase 1. *J. Biol. Chem. 284*, 3998−4008.
(27) Harris, K. M., Flemer, S., Jr., and Hondal, R. J. (2007) Studies on deprotection of cysteine and selenocysteine side chain protecting groups. *J. Pept. Sci. 13*, 81−93.
(28) Flemer, S., Jr., Lacey, B. M., and Hondal, R. J. (2008) Synthesis of peptide substrates for mammalian thioredoxin reductase. *J. Pept. Sci. 14*, 637−647.
(29) Evans, T. C., Jr., Benner, J., and Xu, M.-Q. (1998) Semisynthesis of cytotoxic proteins using a modified protein splicing element. *Protein Sci. 7*, 2256−2264.
(30) Arnér, E. S., Zhong, L., and Holmgren, A. (1999) Preparation and assay of mammalian thioredoxin and thioredoxin reductase. *Methods Enzymol. 300*, 226−239.
(31) Bjornstedt, M., Kumamr, S., Bjorkhem, L., Spyrou, G., and Holmgren, A. (1997) Selenium and the thioredoxin and glutaredoxin systems. *Biomed. Environ. Sci. 10*, 271−279.
(32) Carugo, O., Cemazar, M., Zahariev, S., Hudáky, I., Gáspári, Z., Perczel, A., and Pongor, S. (2003) Vicinal disulfide turns. *Protein Eng. 16*, 637−639.
(33) Singh, R., and Whitesides, G. M. (1990) Degenerate intermolecular thiolate-disulfide interchange involving cyclic five-membered disulfides is faster by $\sim 10^3$ than that involving six- or seven-membered disulfides. *J. Am. Chem. Soc. 112*, 6304−6309.
(34) Bauer, H., Massey, V., Arscott, L. D., Schirmer, R. H., Ballou, D. P., and Williams, C. H., Jr. (2003) The mechanism of high M_r thioredoxin reductase from *Drosophila melanogaster*. *J. Biol. Chem. 278*, 33020−33028.
(35) Burgers, P. M., and Eckstein, F. (1979) Diastereomers of 5′-*O*-adenosyl 3′-*O*-uridyl phophorothioate: Chemical synthesis and enzymatic properties. *Biochemistry 18*, 592−596.
(36) Breslow, R., and Chapman, W. H., Jr. (1996) On the mechanism of action of ribonuclease A: Relevance of enzymatic studies with a *p*-nitrophenylphosphate ester and a thiophosphate ester. *Proc. Natl. Acad. Sci. U.S.A. 93*, 10018−10021.
(37) Herschlag, D. (1994) Ribonuclease revisited: Catalysis via the classical general acid-base mechanism or a triester-like mechanism? *J. Am. Chem. Soc. 116*, 11631−11635.
(38) Messmore, J. M., Fuchs, D. N., and Raines, R. T. (1995) Ribonuclease A: Revealing structure-function relationships with semisynthesis. *J. Am. Chem. Soc. 117*, 8057−8060.
(39) Gerlt, J. A., Kreevoy, M. M., Cleland, W. W., and Frey, P. A. (1997) Understanding enzymic catalysis: The importance of short, strong hydrogen bonds. *Chem. Biol. 4*, 259−267.
(40) Hondal, R. J., and Ruggles, E. L. (2011) Differing views of the role of selenium in thioredoxin reductase. *Amino Acids 41*, 73−89.
(41) Zhong, L., Arnér, E. S., Ljung, J., Aslund, F., and Holmgren, A. (1998) Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. *J. Biol. Chem. 273*, 8581−8591.
(42) Wong, K. K., Vanoni, M. A., and Blanchard, J. S. (1988) Glutathione reductase: Solvent equilibrium and kinetic isotope effects. *Biochemistry 27*, 7091−7096.
(43) Gilbert, H. F. (1990) Molecular and cellular aspects of thiol-disulfide exchange. *Adv. Enzymol. Relat. Areas Mol. Biol. 63*, 69−172.
(44) Gilbert, H. F. (1995) Thiol/disulfide exchange equilibria and disulfide bond stability. *Methods Enzymol. 251*, 8−28.
(45) Gilbert, H. F. (1997) Thiol/disulfide exchange and redox potentials of proteins. In *Bioelectrochemistry of Biomacromolecules* (Lenaz, G., and Milazzo, G., Eds.) pp 256−324, Birkhaeuser, Basel, Switzerland.
(46) Sing, R., and Whitesides, G. M. (1993) Thiol-disulfide interchange. In *Supplement S: The chemistry of sulfur-containing functional groups* (Patai, S., and Rappaport, Z., Eds.) pp 633−658, John Wiley & Sons, New York.
(47) Koshland, D. E., Jr., and Neet, K. E. (1968) The catalytic and regulatory properties of enzymes. *Annu. Rev. Biochem. 37*, 359−411.
(48) Grayson, M., and Janusz, S. J. (2008) Towards a generic model of catalysis. *Bull. Chem. Soc. Ethiop. 22*, 433−440.