BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article
pubs.acs.org/biochemistry

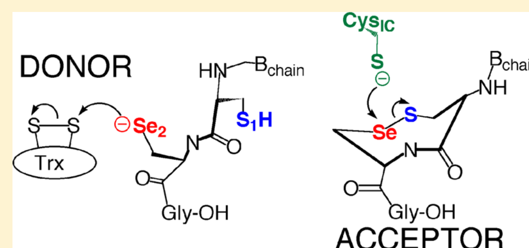
Selenium as an Electron Acceptor during the Catalytic Mechanism of Thioredoxin Reductase

Adam P. Lothrop,[†] Gregg W. Snider,[†] Erik L. Ruggles,[†] Amar S. Patel,[‡] Watson J. Lees,[‡] and Robert J. Hondal*[,†]

[†]Department of Biochemistry, University of Vermont, College of Medicine, 89 Beaumont Avenue, Given Building Room B413, Burlington, Vermont 05405, United States

[‡]Department of Chemistry and Biochemistry, Florida International University, Miami, Florida 33199, United States

ABSTRACT: Mammalian thioredoxin reductase (TR) is a pyridine nucleotide disulfide oxidoreductase that uses the rare amino acid selenocysteine (Sec) in place of the more commonly used amino acid cysteine (Cys) in the redox-active tetrapeptide Gly-Cys-Sec-Gly motif to catalyze thiol/disulfide exchange reactions. Sec can accelerate the rate of these exchange reactions (i) by being a better nucleophile than Cys, (ii) by being a better electrophile than Cys, (iii) by being a better leaving group than Cys, or (iv) by using a combination of all three of these factors, being more chemically reactive than Cys. The role of the selenolate as a nucleophile in the reaction mechanism was recently demonstrated by creating a mutant of human thioredoxin reductase-1 in which the Cys_{497}-Sec_{498} dyad of the C-terminal redox center was mutated to either a Ser_{497}-Cys_{498} dyad or a Cys_{497}-Ser_{498} dyad. Both mutant enzymes were incubated with human thioredoxin (Trx) to determine which mutant formed a mixed disulfide bond complex. Only the mutant containing the Ser_{497}-Cys_{498} dyad formed a complex, and this structure has been determined by X-ray crystallography [Fritz-Wolf, K., Kehr, S., Stumpf, M., Rahlfs, S., and Becker, K. (2011) Crystal structure of the human thioredoxin reductase-thioredoxin complex. *Nat. Commun.* *2*, 383]. This experimental observation most likely means that the selenolate is the nucleophile initially attacking the disulfide bond of Trx because a complex resulted only when Cys was present in the second position of the dyad. As a nucleophile, the selenolate of Sec helps to accelerate the rate of this exchange reaction relative to Cys in the Sec → Cys mutant enzyme. Another thiol/disulfide exchange reaction that occurs in the enzymatic cycle of the enzyme is the transfer of electrons from the thiolate of the interchange Cys residue of the N-terminal redox center to the eight-membered selenosulfide ring of the C-terminal redox center. The selenium atom of the selenosulfide could accelerate this exchange reaction by being a good leaving group (attack at the sulfur atom) or by being a good electrophile (attack at the selenium atom). Here we provide strong evidence that the selenium atom is attacked in this exchange step. This was shown by creating a mutant enzyme containing a Gly-Gly-Sec_{coo-} motif that had 0.5% of the activity of the wild-type enzyme. This mutant lacks the adjacent, resolving Cys residue, which acts by attacking the mixed selenosulfide bond that occurs between the enzyme and substrate. A similar result was obtained when Sec was replaced with homocysteine. These results highlight the role of selenium as an electron acceptor in the catalytic mechanism of thioredoxin reductase as well as its established role as a donor of an electron to the substrate.



Mammalian thioredoxin reductase (TR) is a pyridine nucleotide disulfide oxidoreductase that uses the rare amino acid selenocysteine (Sec, U) in place of the more commonly used amino acid cysteine (Cys) in its catalysis of the reduction of its major target protein, thioredoxin (Trx), and a variety of small molecule substrates.[1−10] The thioredoxin system, comprised of TR, Trx, and NADPH, is one of the major antioxidant systems in mammalian cells.[9]

The use of a selenium (Se) atom in place of a sulfur (S) atom in TR and other selenoenzymes is most likely due to important physicochemical differences between the two residues and confers some biological advantage to the organism containing the selenoenzyme. The physicochemical advantage given to an enzyme that uses Sec instead of Cys is the subject of ongoing debate in the field.[11−13] Most selenoenzymes studied to date are oxidoreductases that are involved in thiol/disulfide exchange reactions in which Sec replaces Cys. In such cases, the presence of selenium in the form of a selenolate or selenosulfide can accelerate the rate of the exchange reaction by being (i) a better nucleophile than a thiolate, (ii) a better electrophile than a disulfide, (iii) a better leaving group than a thiolate, or (iv) a more chemically reactive species compared to sulfur in the form of a thiolate or disulfide using a combination of all three of these factors.

Selenocysteine participates in the reaction mechanism of TR in two key thiol/disulfide exchange reactions as shown in Figure 1. First, the selenolate of Sec acts as the donor of electrons to the substrate, the disulfide bond of Trx (Figure

Received: May 26, 2013
Revised: January 4, 2014
Published: January 15, 2014

 © 2014 American Chemical Society dx.doi.org/10.1021/bi400658g | *Biochemistry* 2014, 53, 654−663

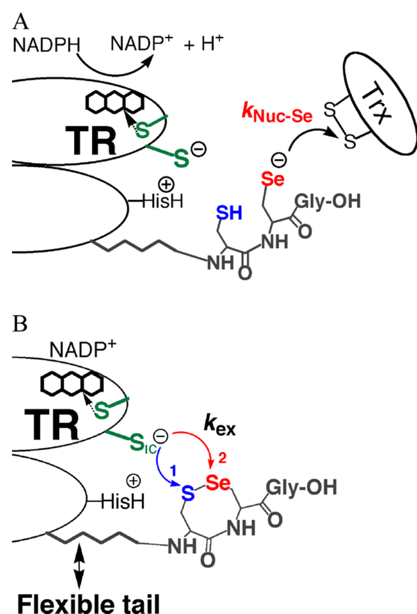


Figure 1. Mechanistic roles of Se in the TR reaction mechanism as a donor or acceptor of electrons. In the TR reaction mechanism, electrons flow from NADPH to the enzyme-bound flavin. The reduced flavin coenzyme then reduces the N-terminal redox center, consisting of Cys_{CT} and Cys_{IC} (colored green). Cys_{IC} then attacks the selenosulfide bond of the C-terminal Cys-Sec dyad, forming either a mixed disulfide (path 1) or a mixed selenosulfide (path 2). Cys_{CT} then resolves the mixed disulfide/selenosulfide, re-forming the N-terminal disulfide between Cys_{CT} and Cys_{IC}. For a complete description of the mechanism, please see ref 14. Two equivalents of NADPH are required to produce the active EH_4 form. The EH_4 form is the active form of the enzyme that reduces Trx as shown by Williams and co-workers.[14] (A) Se donates electrons to Trx in an initial nucleophilic attack step. The individual rate constant for this step is given as $k_{Nuc\text{-}Se}$. (B) Se can accelerate the thiol/disulfide exchange step that occurs between the two reaction centers either by acting as a good leaving group (arrow 1) or by acting as a good electrophile (arrow 2). The individual rate constant for this step is given as k_{ex}. Mutating Sec to Cys may impair the enzyme's ability to either donate or accept electrons as discussed in the text.

1A), and second, as part of the selenosulfide bond found in the C-terminus of the enzyme, selenium takes part in accepting electrons from the N-terminal redox center of TR (Figure 1B). It is well-established that selenols and selenolates are better nucleophiles than are thiols and thiolates,[15−18] and because Sec was first discovered in TR, the selenolate has been the presumptive nucleophile for the initial attack on a substrate disulfide bond in the TR mechanism.[15] Experimental evidence of this includes a large decrease in reaction velocity when Sec is replaced by Cys,[15,19,20] as well as recent crystallographic data in which TR was crystallized in a mixed disulfide complex with a mutant form of human Trx1 (hTrx1).[21] In this latter experiment, the C-terminal Cys_{497}-Sec_{498} redox dyad was mutated to a Ser_{497}-Cys_{498} dyad, and this TR mutant was incubated with the C35S/C73S mutant of hTrx1. The result was a mixed disulfide-bonded complex between Cys498 of human TR (hTR) and Cys32 of hTrx1. This is strong evidence that Sec498 of hTR in the wild-type (WT) enzyme is the attacking nucleophile in thiol/disulfide exchange reactions involving substrates such as the one shown in Figure 1A.

After reducing its substrate, the C-terminal Cys-Sec dyad of TR becomes oxidized and forms an eight-membered ring structure (Figure 1B). This vicinal selenosulfide bond then undergoes another thiol/disulfide exchange reaction with the N-terminal redox center that is composed of an interchange Cys residue (Cys_{IC}) and a charge-transfer Cys (Cys_{CT}) residue that interacts with the flavin coenzyme. As explained in the legend of Figure 1B, the thiolate of Cys_{IC} can attack either the sulfur (path 1) or the selenium (path 2) of the selenosulfide bond. Unlike the thiol/disulfide exchange step involving Trx, there is no X-ray crystal structure of a trapped mixed disulfide intermediate between the C-terminal redox center and the N-terminal redox center. Attempts have been made to model this thiol/disulfide exchange step in crystal structures of various high-M_r TR enzymes.[22−24] These studies are inconclusive because they show that either path 1 or path 2 is a feasible route of attack *depending* on how the C-terminus is modeled into the active site.

In our early work, we had argued for path 1 because of the belief that the selenolate of Sec would act as a superior leaving group in this exchange reaction.[24,25] Path 1 can thus be described as a "Se as a leaving group" model. Later, we began to favor path 2, which involves attack at the selenium of the selenosulfide by Cys_{IC}, and this can be described as the "Se as an electrophile" model. This model was supported by experimental data that showed that the truncated TR, missing the C-terminal tail, would only reduce highly electrophilic, small molecule substrates, irrespective of whether they contained a low-pK_a leaving group.[6,26] This model is also supported by recent theoretical and experimental studies that show that attack at the selenium in a selenosulfide is highly favored over attack at the sulfur in thiol/disulfide exchange reactions.[27,28] The high electrophilicity of selenide relative to that of sulfide has been recognized in the field of chemistry[29] but is a largely unrecognized property in the biochemical literature.

In this report, we resolve the question of attack at Se or S in a selenosulfide by performing Brønsted analyses of synthetic, disulfide-containing substrates in which the pK_a of the leaving group thiol was varied by changing the substituent at the *para* position of the aromatic ring. In addition, we have constructed a mutant of TR with either a single Sec residue or a single sulfhydryl group in place of the Cys-Sec redox dyad in the C-terminal redox center. The data from these mutants show that the second position of the C-terminal redox dyad, occupied by a Sec residue, is responsible for both donating electrons to Trx and accepting them (from Cys_{IC}, path 2) in the thiol/disulfide exchange reaction that occurs between the N- and C-terminal redox centers. The results reported here highlight how the enzyme can use selenium to accelerate thiol/disulfide exchange reactions.

■ MATERIALS AND METHODS

Materials. NADPH was purchased from AppliChem (Darmstadt, Germany). Dithionitrobenzoic acid (DTNB), sodium selenite, and DEAE resin were all obtained from Sigma-Aldrich (St. Louis, MO). Phenyl Sepharose resin was from Pharmacia-Amersham Biosciences (Uppsala, Sweden). Microcon Ultracel YM-50 ultrafiltration devices from Millipore (Billerica, MA) were used for concentrating enzyme samples. Resin for peptide synthesis (2-chlorotritylchloride) 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). Primers for mTR3 mutants were from IDT (Coralville, IA).

Plasmid pTYB3 and restriction enzymes were from New England Biolabs (Ipswich, MA). The production and purification of the recombinant and semisynthetic enzymes used in this study have been previously reported.[6,19,24,25,30] The selenium content of the wild-type (WT) semisynthetic enzyme is 91% as reported in ref 19. 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 or Acros Organics (Morris Plains, NJ). Aryl disulfides were prepared by Watson Lees and others as described in refs 31−35.

Peptide Synthesis. All peptides in this study were synthesized on 2-chlorotritylchloride resin using standard Fmoc chemistry as previously detailed.[19,25,36] Peptides were cleaved from the resin using trifluoroacetic acid (TFA) containing triisopropylsilane and water in a 96:2:2 ratio. The cleavage volume was reduced by evaporation under a stream of N_2 or air, and the peptides were then precipitated in ice-cold diethyl ether. Once dry, the peptides were redissolved in a 90:10 (v/v) water/acetonitrile mixture and lyophilized. The freeze-dried peptide was then analyzed by both MALDI-TOF mass spectrometry and analytical HPLC to judge the composition and purity.

Peptides **I**−**IV** (Table 1) containing a mixed aryl disulfide bond were constructed by first synthesizing a peptide corresponding to the sequence of the C-terminus of mTR, with the Sec residue replaced with a Gly residue (H-PTVTGCGG-OH). This peptide and a *para*-substituted aryl disulfide compound (1:3 molar ratio) were dissolved in 50 mM potassium phosphate buffer (pH 8.0), and then the mixture was stirred at room temperature for 16 h. Peptides **I**−**IV** were isolated by HPLC, analyzed by MALDI-TOF MS, and lyophilized for later use.

Production of Recombinant and Semisynthetic Mutants of Mouse Mitochondrial TR (mTR3). A brief description of the nomenclature used in Tables 2 and 3 (and throughout) is warranted here. The C-terminal redox-active tetrapeptide of mTR3 contains the amino acids H-Gly-Cys-Sec-Gly-OH. In Table 2, we abbreviate the main body of the enzyme as mTR3 and the C-terminal tetrapeptide as GCUG. Thus, the WT enzyme is abbreviated as mTR3-GCUG throughout this report. Mutants are abbreviated as mTR3-$aa_1aa_2aa_3aa_4$ (using one-letter codes for the amino acids). The production of both the full-length WT enzyme and semi-synthetic mutant enzymes by intein-mediated peptide ligation (IPL) has been previously described.[19] Briefly, mTR3 missing the final three C-terminal residues (mTR3Δ3) is expressed as an intein−chitin binding domain (CBD) fusion protein in *Escherichia coli* cells and then bound to chitin agarose beads. Enzyme **1** was produced by cleaving a truncated enzyme−intein fusion protein with 50 mM *N*-methylmercaptoacetamide (NMA) in the presence of peptide H-Cys-Sec-Gly-OH to effect ligation of the peptide to the thioester-tagged enzymes (Tables 2 and 3).[19] Enzyme **2**, the Sec → Cys mutant of mTR, was produced using standard recombinant methods previously reported by us.[19] Cleavage of the truncated enzyme−intein fusion protein with either L-cysteine or L-homocysteine in the absence of NMA yielded enzymes **3** and **4**. L-Homocysteine was generated from a solution of L-homocystine (25 mM) dissolved in 50 mM MOPS buffer (pH 7.0) with 150 mM NaCl and 75 mM DTT. The pH of the solution was adjusted to 10 to improve the homocysteine solubility and then reduced to 7.9 for optimal cleavage of the enzyme from the intein. After incubation for 30 min, this cleavage buffer was added to the chitin resin-bound enzyme, and the mixture was gently agitated overnight at 4 °C. Enzyme **5** was produced by treating the mTR3Δ3−intein fusion protein with 50 mM L-selenocysteine. L-Selenocysteine was produced by reducing L-selenocystine with a 3-fold excess of sodium borohydride in water for 15 min. The excess sodium borohydride was quenched by addition of acetic acid, after which the solution was brought to pH 7.0 by addition of 500 mM potassium phosphate buffer. This solution was then immediately applied to the bound fusion protein on the chitin resin.

The mTR3Δ3 fusion protein construct was modified to include an additional codon (GGT, Gly) between mTR3 and the intein−CBD fusion protein to give a mutant in which Cys_1 was mutated to Gly, resulting in an mTR3Δ2 fusion construct. This construct was cleaved from the intein using either L-cysteine or L-homocysteine to generate enzymes **6** and **7**. Enzyme **8** was produced by treating the mTRΔ2−intein fusion protein with 50 mM selenocysteine using a procedure identical to that described for enzyme **5**.

Truncated mTR enzymes missing either the final three C-terminal amino acids or the final eight C-terminal amino acids, mTR3Δ3 or mTR3Δ8, respectively (enzyme **9** or **10**, respectively), were produced as reported by us previously.[19,25,30] All enzyme constructs were purified using hydrophobic and anion exchange chromatography as described previously.[19,25,30] After purified enzymes had been concentrated using ultrafiltration, homodimeric enzyme concentrations were determined by measuring the absorbance of flavin adenine dinucleotide (FAD) at 460 nm using an extinction coefficient of 11.3 mM^{-1} cm^{-1} and a stoichiometric ratio of 2 mol of FAD/mol of dimeric enzyme.

Enzymatic Assays of TR. The assays for Trx, DTNB, and selenocystine as substrates of TR have been previously described.[6,19,24,25,30,37] 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. DTNB assays were conducted with 100 mM potassium phosphate buffer (pH 7.0), 10 mM EDTA, 200 μM NADPH, and various concentrations of DTNB. Assays with selenocystine as the substrate contained 500 mM potassium phosphate (pH 7.0), 10 mM EDTA, 200 μM NADPH, and various concentrations of selenocystine.

Brønsted Analysis of Aryl Disulfide Peptide Substrates. Assays with arylthiol peptide substrates **I**−**IV** in Table 1 contained 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 150 μM NADPH, and various concentrations of peptide substrates (between 50 and 2000 μM). The assay was initiated by the addition of 96.4 nM mTR3Δ8. The rate constant was calculated as the slope from a plot of the rate of the reaction (micromolar per minute) versus substrate concentration. A Brønsted plot was then constructed by plotting the log of the rate constant versus the pK_a of the thiol of the peptide substrate.

RESULTS AND DISCUSSION

Brønsted Analysis of Mixed Aryl-Peptide Disulfide Substrates Does Not Support the Leaving Group Model. To assess whether our original Se as a leaving group model was correct for the thiol/disulfide exchange reaction shown in Figure 1B, we assayed enzyme mTR3Δ8, which lacks the C-

terminal redox motif, for activity using synthesized mixed aryl-peptide disulfide substrates. The aryl group contained a *para* substituent that allowed for variability of the arylthiol pK_a in the range of 5.7–6.6 (the atom labeled S_2 in Figure 2). The advantage of the experimental design shown in Figure 2 is that it allows for the study of the kinetics of the thiol/disulfide exchange step that occurs between N- and C-terminal redox centers. We refer to this step simply as "the exchange step" hereafter.

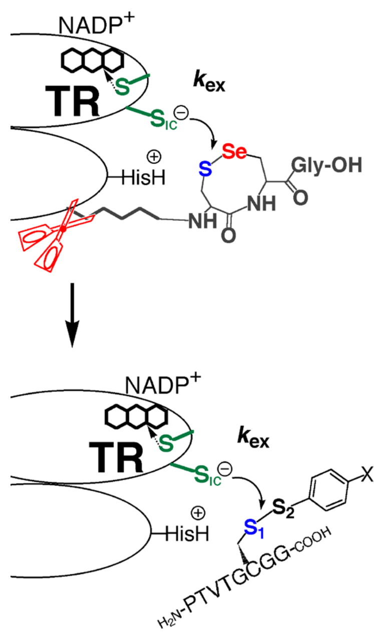


Figure 2. Strategy for isolating the exchange step in the reaction mechanism. Our previous studies as well as this study make use of a truncated enzyme missing the C-terminal redox center in conjunction with small molecule substrates to gain a better understanding of the exchange step in the mechanism (represented by rate constant k_{ex}). Here, we have constructed peptides in which Sec_2 is replaced with a *para*-substituted arylthiol group that allows us to alter the pK_a of S_2 of the product, to measure the dependency of k_{ex} on leaving group pK_a.

The structure, pK_a values, and specific disulfide-reductase activities for these peptide substrates (**I–IV**) using mTR3Δ8 as the enzyme catalyst are given in Table 1. We note that although we used the symbol k_{ex} to denote the individual rate constant of the exchange step in Figure 2, we did not make this measurement. Rather, we report a specific disulfide-reductase activity in Table 1, which is a composite of all of the individual steps on the path needed to generate the product of the reaction.

However, a rate constant for each peptide substrate was determined by plotting the rate of the steady-state reaction (micromolar per minute) versus peptide concentration (micromolar). The slope of the resulting plot yielded the rate constant k. A plot of log k versus pK_a (Brønsted plot) shows that the rate increases with an increase in pK_a as shown in Figure 3. The slope of the line in this plot is the Brønsted coefficient, β, and has a value of 1.1. A high, positive value of β such as this is interpreted to mean either that there is positive charge on S_2 in the transition state or that there is a loss of negative charge on S_2 in the transition state.[42] These data do not support the Se as a leaving group model. If the exchange step in Figure 1B was dominated by the need for a low-pK_a leaving group, the Brønsted coefficient in Figure 3 would be large and negative (i.e., −1). The fact that the experimentally determined value of β was large and positive is interpreted by us to mean that the -S_1–S_2- bond becomes highly polarized in the transition state, with electron density moving away from S_2 toward S_1 of the arylthiol peptide substrate. If the aryl-peptide disulfide substrates are accepted as a reasonable model for the C-terminal redox center of the enzyme, this would mean that in the native enzyme the -S–Se- bond becomes polarized to allow the Se atom to accept electrons more easily from Cys_{IC} during the exchange step. Thus, the analysis of the Brønsted data supports the Se as an electrophile model mentioned in the

Table 1. Activity of mTRΔ8 toward Mammalian Peptide-Aryl Disulfide Substrates

Peptide Number	Peptides I – IV (S_1–S_2–C₆H₄–X; H₂N-PTVTGCGG-COOH)	pK_a of S_2	Activity at 1 mM peptide (mol NADPH/min/mol enzyme)	Relative Activity
I	X = SO_3H	5.7[a]	12.5	2.16
II	X = COOH	5.95[a]	5.8	1
III	X = CH_2OH	6.4[a]	16.4	2.83
IV	X = CH_2COOH	6.6[a]	136.6	**23.6**
V	S–Se, Gly-COOH, NH, O; H₂N-PTVTG–NH, O	Se ~5.2[b]	177	**30.5**
VI	S–S, Gly-COOH, NH, O; H₂N-PTVTG–NH, O	~8.3[c]	0.09	0.015
VII	S–S_2, Gly-COOH, O, NH_2; H₂N-PTVTG–NH, COOH	~8.3[c]	0.03	0.005
VIII	S–Se, Gly-COOH, O, NH_2; H₂N-PTVTG–NH, COOH	Se ~5.2[b]	6.75	1.16
DTNB	HOOC, COOH; O_2N–C₆H₃–S–S–C₆H₃–NO_2	4.75[d]	2000	345

[a]Taken from ref 38. [b]Taken from ref 39. [c]Taken from ref 40. [d]Taken from ref 41. The data reported here for peptides **V–VIII** and **DTNB** was taken from ref 25.

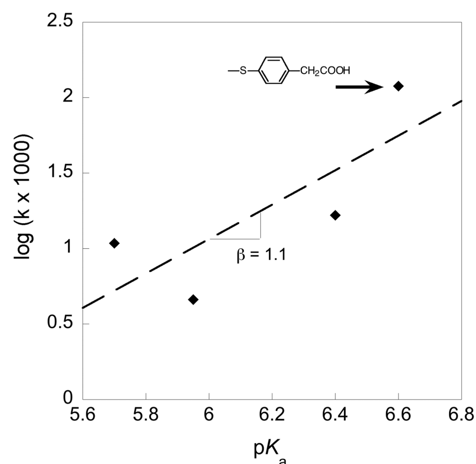


Figure 3. Brønsted plot for the truncated enzyme with peptide disulfide substrates. The plot shows that over this range of pK_a values, high disulfide reductase activity is correlated with increase in thiol pK_a with a Brønsted coefficient of 1.1 (slope). The position of peptide **IV** is indicated by the arrow.

dx.doi.org/10.1021/bi400658g | Biochemistry 2014, 53, 654–663

introductory section. We note that our analysis assumes that dissociation of both products that result from cleavage of the disulfide bond of the aryl-peptide disulfide substrate is not too dissimilar from the dissociation of the C-terminal redox center that is covalently bound to the enzyme. As discussed in the next section, the concept of bond polarization can explain why some disulfides can be used as substrates by the N-terminal redox center and others cannot.

In the Absence of Se, Some Disulfides Are Good Substrates for the N-Terminal Redox Center: An Explanation. A good example illustrating how bond polarization (electrophilicity) can accelerate the rate of the exchange reaction between the N-terminal redox center of mTR3Δ8 and disulfide substrates is the comparison of two *symmetric*, linear disulfides such as DTNB and cystine. The disulfide bond of DTNB is highly polarized (activated) because of the attachment of strong, symmetrical electron-withdrawing nitroaryl groups and is turned over 11−14-fold faster than peptides **IV** and **V** (Table 1). Symmetrical, nonpolarized disulfides, such as cystine, are extremely poor substrates for the truncated enzyme. For the purposes of direct comparison, the activity of mTR3Δ8 toward 200 μM DTNB is 420 mol of NADPH min^{-1} (mol of enzyme)$^{-1}$, while at the same concentration of cystine, the activity is 0.1 mol of NADPH min^{-1} (mol of enzyme)$^{-1}$, a 4200-fold difference.

The Se as a leaving group model predicted that mTR3Δ8 would reduce peptide **IV** (highest pK_a) with the slowest rate and peptide **I** (lowest pK_a) with the fastest rate. The opposite result was found experimentally (Table 1). The mTR3 enzyme has nearly 24-fold higher activity with peptide **IV** than with peptide **II**, in which the arylthio group has a pK_a that is lower by 0.65 pK units. In fact, the activity with peptide **IV** was nearly as high as that with peptide **V**, which contains the "native", vicinal selenosulfide bond, identical to that which is found in the C-terminal tail of the enzyme (the result with **V** was originally reported in ref 25).

Upon closer inspection of the structure of **IV**, we realized that it can mimic the physicochemical properties of the native peptide **V** in two ways. First, peptide **IV** can be held rigidly in the active site, just as is true for any substrate that is efficiently turned over in a typical enzyme active site. We suggest that the reason for peptide **IV** being rigidly held is that the *p*-carboxylate group is the "correct" distance from S_2, allowing it to form an ionic interaction with a basic group on the enzyme as shown in Figure 4. The geometric similarity between **IV** and the native peptide is further explained in the legend of Figure 5. Even though **IV** is a linear disulfide, the ability to be held rigidly in the active site would mimic the geometry of the eight-membered ring in the native peptide. Second, the S_1−S_2 bond is polarized because of the presence of the *para*-substituted aryl group and would be further polarized if S_1 were near a positive charge in the active site, such as His463′,[a] which acts as an acid/base catalyst during the enzymatic reaction cycle. Both of these factors would help to make S_2 electron deficient (make S_2 electrophilic) and accelerate the thiol/disulfide exchange reaction between the N-terminal redox center and the substrate.

The correct active site geometry is not the only requirement for disulfide substrates to be turned over by mTR3Δ8 because peptide **VII** meets the same distance criteria between S_2 and the C-terminal carboxylate as peptide **IV** (both asymmetric, linear disulfides). However, **IV** is turned over ∼4500-fold faster than **VII** by mTR3Δ8. This large difference in rate acceleration can

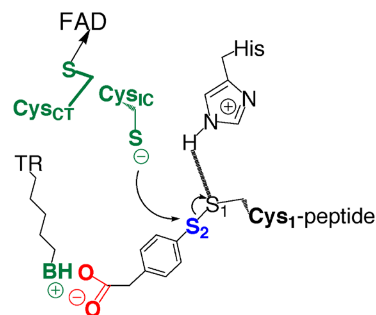


Figure 4. Peptide **IV** can be held rigidly in the active site and activated for the exchange reaction. The correct placement of the peptide disulfide in **IV** relative to the N-terminal active site could serve to "electrophilically activate" the disulfide for attack by Cys_{IC} by positioning atom S_1 of the disulfide near the positively charged imidazolium group of His463′, inducing a strong polarization in the disulfide bond of **IV**. This polarization would be made possible by tight binding of the substrate in the active site because of an interaction between the negatively charged phenylacetate group (colored red) and a basic group on the enzyme (colored green). The basic group on the enzyme has been predicted to be either Lys29 in the rat TR1 structure (equivalent to Lys29 in the mouse TR3 enzyme) or Arg351 in a model of the human TR−Trx complex structure (equivalent to Arg342 in the mouse TR3 enzyme).[43,44] This suggests how a similar exchange reaction is accelerated in the Cys ortholog. For the sake of simplicity, we do not show the interaction between FAD and $NADP^+$.

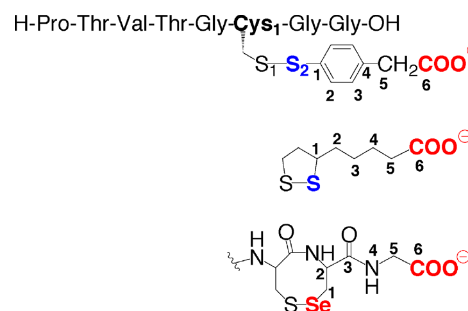


Figure 5. We note that the distance between S_2 of the disulfide of **IV** and the carboxylate group (colored red) is similar to the distance between the Se atom of the native peptide and the C-terminal carboxylate group (five intervening carbon atoms). This similarity could further explain why the disulfide of **IV** is turned over at a rate similar to that of the selenosulfide of peptide **V**. This is also similar to the distance between the carboxylate group of lipoic acid and the sulfur atom of the ring. We have shown previously that lipoic acid is a surrogate substrate for the C-terminal redox center.[6] This structural similarity of the various substrates is underscored by the evolutionary relationship between TR and lipoamide dehydrogenase, another pyridine nucleotide disulfide oxidoreductase that reduces lipoyl-disulfide groups.

be explained if the -S_1−S_2- disulfide bond of **IV** is highly polarized as we posit. If the S atom of **VII** is removed and replaced by Se as is the case for **VIII**, the rate increases 225-fold. This latter number is in the range of the fold decrease in activity when Sec is mutated to Cys in TR and other selenoenzymes.[20,45] A reasonable conclusion from these data is that Se confers polarization to a -S−Se- bond, and this bond polarization is responsible for the acceleration of the rate in **VIII** compared to that in **VII**.

Further support for this polarization hypothesis is given by comparing the turnover rates of peptide substrates in Table 1

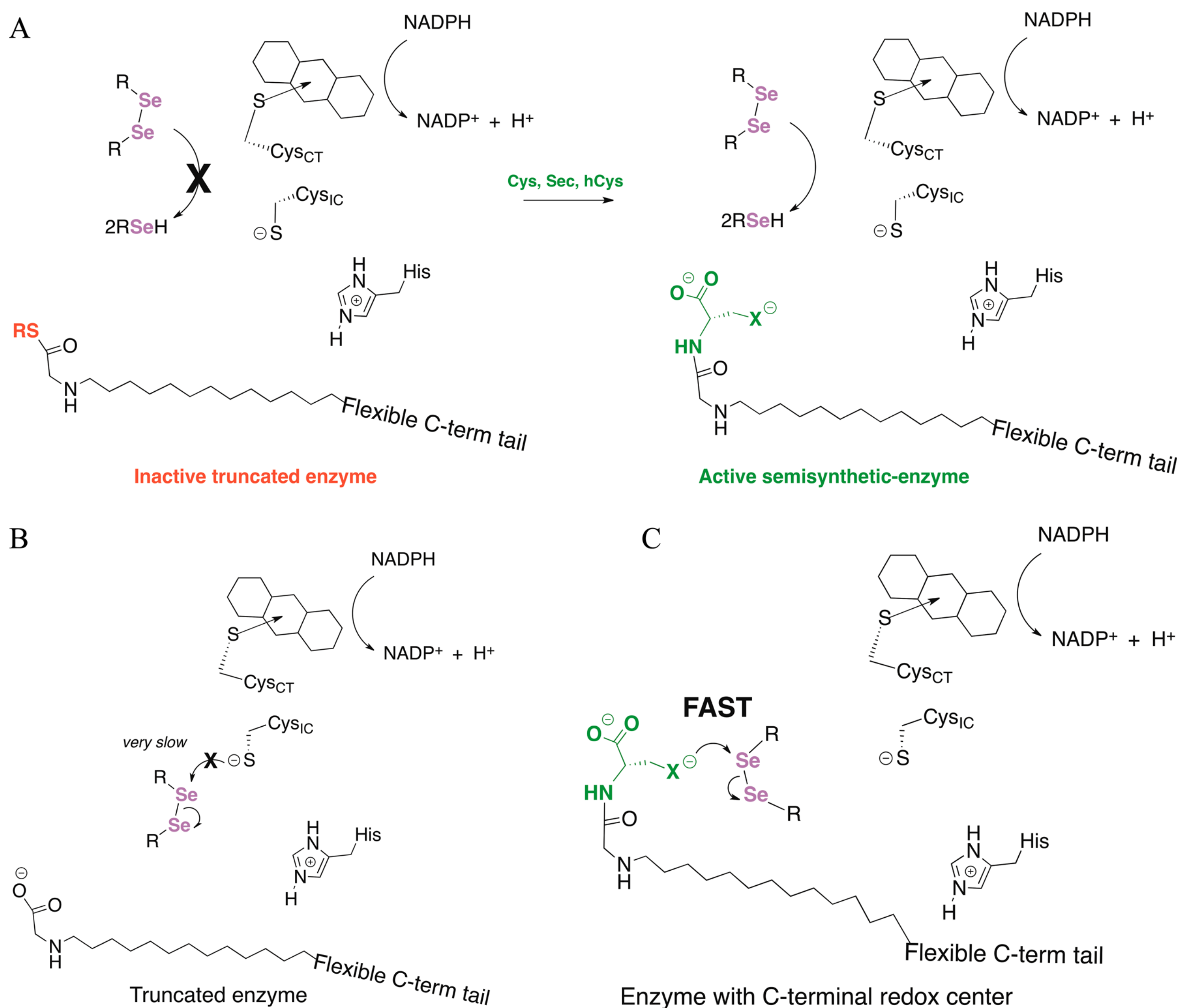


Figure 6. Selenocystine-reductase activity of mTR3 as a semiquantitative measure of ligation efficiency. (A) The truncated enzyme lacking the Cys_1-Sec_2 dyad has very low selenocystine-reductase activity as reported by us previously.[6] In the experiments reported here, we constructed mutants containing a single redox-active residue (Cys, hCys, or Sec) by using these amino acids as reagents that would simultaneously undergo a transthiolesterification reaction with the thioester-tagged mTR3 enzyme produced as an intein fusion protein and subsequently become ligated to the C-terminal end. Cleavage of mTR3Δ3 with either Cys, hCys, or Sec results in the production of enzyme **3**, **4**, or **5**, respectively (Table 2). Similarly, cleavage of mTR3Δ2 with either Cys, hCys, or Sec results in the production of enzyme **6**, **7**, or **8**, respectively. All of these enzymes have significantly higher selenocystine-reductase activity than the truncated enzyme (Table 2), demonstrating that cleavage and ligation were successful. (B) The truncated enzyme cannot reduce selenocystine because of an apparent lack of ability of the N-terminal redox center to catalyze this reduction. (C) Reduction of selenocystine by mTR3 can be achieved with either a fully intact C-terminal redox center as in the case of enzymes **1** and **2** or a partially intact C-terminal redox center containing only a single redox-active residue (enzymes **3**−**8**). While we do not know complete mechanistic details for certain, the most likely explanation is that the C-terminal redox center is required for initiating attack on the substrate diselenide bond.

that differ by only a single feature. For example, the **V**:**VI** turnover ratio shows that bond polarization contributes a factor of ∼2000 to rate acceleration. Comparison of the activities of **V**/**VIII** and **VII**/**VIII** pairs implies that a combination of correct distance and geometry contributes a factor of 10−25 to the exchange rate, while polarization (electrophilicity) contributes a factor of 200 for the mammalian enzyme. Please note that peptides **VI** and **VII** are both turned over at nearly the same slow rate, which shows the dramatic effect that the loss of polarization in the disulfide bond caused by substitution of S for Se has on the exchange step. This analysis of the data in Table 1 supports the Se as an electrophile model and supports path 2 as being the correct pathway for the exchange reaction shown in Figure 1B.

Functional Assay for Assessing Peptide Incorporation in IPL. Enzymes **3**−**8** were constructed by using IPL.[46] This method allows us to incorporate Sec, hCys, and other non-natural amino acids into TR to investigate the function of the C-terminal redox center as done here and reported by us previously.[19,24,30] IPL usually involves ligation of a peptide containing an N-terminal Cys, Sec, or hCys residue to a thioester-tagged protein.[46] However, here we are using a *single* amino acid instead of a peptide to achieve the same goal. The use of Cys, Sec, or hCys as a cleavage/ligation reagent results in the addition of a single amino acid to the C-terminal end of mTR3 as shown in Figure 6A. One possible side reaction of the thioester is hydrolysis, which would result in protein eluting from the column without the addition of the added amino acid. To test whether cleavage of the thioester-tagged TR was due to

dx.doi.org/10.1021/bi400658g | Biochemistry 2014, 53, 654−663

Table 2. Selenocystine Reductase Activities of WT mTR3 and Mutant Enzymes

enzyme	enzyme number	k_{cat} (min^{-1})	K_m (μM)	activity at 91 μM [mol of NADPH min^{-1} (mol of enzyme)$^{-1}$]
mTR-GCUG	**1**	1403.6 ± 128	341.2 ± 59.0	295 ± 20
mTR-GCCG	**2**	681.7 ± 91.5	791.3 ± 151.7	78 ± 3
mTR-GC	**3**	NA[a]	NA[a]	12.5 ± 2
mTR-Gh**C**	**4**	NA[a]	NA[a]	8 ± 1
mTR-GU	**5**	NA[a]	NA[a]	176 ± 4
mTR-GGC	**6**	NA[a]	NA[a]	12 ± 1
mTR-GGh**C**	**7**	NA[a]	NA[a]	16 ± 0.1
mTR-GGU	**8**	194.5 ± 7	10.4 ± 2	124 ± 9
mTR-G	**9**	NA[a]	NA[a]	1.4 ± 0.2
mTRΔ8	**10**	NA[a]	NA[a]	0.6 ± 0.1

[a]Not applicable.

the addition of amino acid rather than hydrolysis of the thioester, a control experiment was performed for enzymes **3**−**8** to measure the amount of cleavage in the absence of an amino acid from the cleavage buffer. These experiments yielded an eluent from the chitin column with no measurable selenocystine-reductase activity, which is evidence that little or no hydrolysis of the thioester occurs during incubation with the cleavage buffer containing the single amino acid.

In ligation experiments of the type performed here, it is desirable to achieve complete ligation of the peptide (amino acid) to the thioester-tagged protein. To test the efficiency of the ligation of the amino acid to thioester-tagged mTR3, we have devised an assay that allows us to make a semiquantitative assessment of ligation efficiency as shown in panels B and C of Figure 6. This assay uses the reduction of selenocystine as an alternative substrate in place of Trx. We have previously reported that the Sec → Cys mutant of mitochondrial TR and any variant in which the C-terminal redox center contains either a sulfhydryl or selenol, as is the case with enzymes **3**−**8**, will reduce selenocystine as the data in Table 2 shows.[6] Thus, this assay becomes a simple test that allowed us to determine whether the C-terminal amino acid that we added in the cleavage buffer is present. If the amino acid were not ligated to the enzyme, the mutant should have selenocystine reductase activity similar to that of the truncated enzyme (enzyme **10**). The data in Table 2 indicate that the cleavage/ligation procedure was successful in adding Cys, Sec, or hCys to the C-terminus of TR. A second conclusion that can be drawn from the data in Table 2 is that enzymes **3**−**8**, with only partially intact C-terminal redox centers, are able to catalyze the same two thiol/disulfide exchange reactions that are shown in panels A and B of Figure 1 except that selenocystine has replaced Trx as the substrate. As discussed in the next section, only enzymes **7** and **8** can also reduce Trx.

Evidence That Selenium both Donates and Accepts Electrons in the Trx-Reductase Mechanism. The selenium atom of TR acts as the electron donor to the disulfide bond of Trx in both the selenium as a leaving group model (path 1 in Figure 1B) and the selenium as an electrophile model (path 2 in Figure 1B). We therefore predicted that a mutant of mTR3 containing only a single Sec residue in the C-terminal redox center would have Trx-reductase activity only if the single Sec residue was in the second position of the C-terminal Cys_1-Sec_2 redox dyad and selenium acted as the acceptor of electrons from the N-terminal redox center.[b] This prediction was tested by creating mutant enzymes **5** and **8** and assaying these enzymes for Trx-reductase activity. As can be seen by the data listed in Table 3, while enzyme **8** has only 0.5% of the Trx-

Table 3. Trx-Reductase Activities of WT mTR3 and Mutant Enzymes

enzyme	enzyme number	k_{cat} (min^{-1})	K_m (μM)
mTR-GCUG	**1**	2220 ± 78	67.6 ± 6
mTR-GCCG	**2**	4 ± 0.1	49 ± 3
mTR-GC	**3**	ND[a]	ND[a]
mTR-Gh**C**	**4**	ND[a]	ND[a]
mTR-GU	**5**	ND[a]	ND[a]
mTR-GG**C**	**6**	ND[a]	ND[a]
mTR-GGh**C**	**7**	4 ± 0.1	42 ± 3
mTR-GGU	**8**	11.6 ± 0.3	8 ± 1
mTR-G	**9**	ND[a]	ND[a]
mTRΔ8	**10**	ND[a]	ND[a]

[a]Not detectable.

reductase activity of the WT enzyme, it has more than twice the Trx-reductase activity as the Sec → Cys mutant of mTR3 (enzyme **2**). Enzyme **5** has no detectable Trx-reductase activity in comparison. This experimental result provides strong evidence for the selenium as an electrophile model and also confirms the role of selenolate as the electron donor to substrate provided by the X-ray crystal structure of the mutant TR−Trx complex.[21]

As a further test of this hypothesis, we also considered whether selenolate could be acting as a leaving group in the mixed selenosulfide bond of the TR−Trx complex in mutant enzyme **8**. In such a case, the thiolate of Cys_{IC} would attack the sulfur atom of the mixed selenosulfide bond and the reaction in mutant **8** would be fast relative to that with mutant **6** because of the superior leaving group ability of selenium. This possibility was tested by replacing each residue of the Cys_1-Sec_2 redox dyad with homocysteine (hCys, hC), while leaving the other position vacant to create mutants **4** and **7**, respectively. These two mutants are analogous to mutants **5** and **8** in design and construction. The thiolates of the Cys and hCys residues should have identical leaving group abilities and have similar nucleophilic character. A key difference is that the introduction of a hCys residue lengthens the distance between Cα and the sulfur atom by ∼3 Å relative to Cys. This added side chain length and flexibility should make the sulfur atom of hCys more accessible to the thiolate of Cys_{IC} for attack in a mixed disulfide complex between mutant TR and Trx. The results show that Trx-reductase activity is only present when the second position of the dyad is replaced with hCys [enzyme **7** (Table 3)]. This result confirms that the second position of the redox dyad is responsible for both accepting electrons from the N-terminal redox center and donating electrons to the substrate, whether it

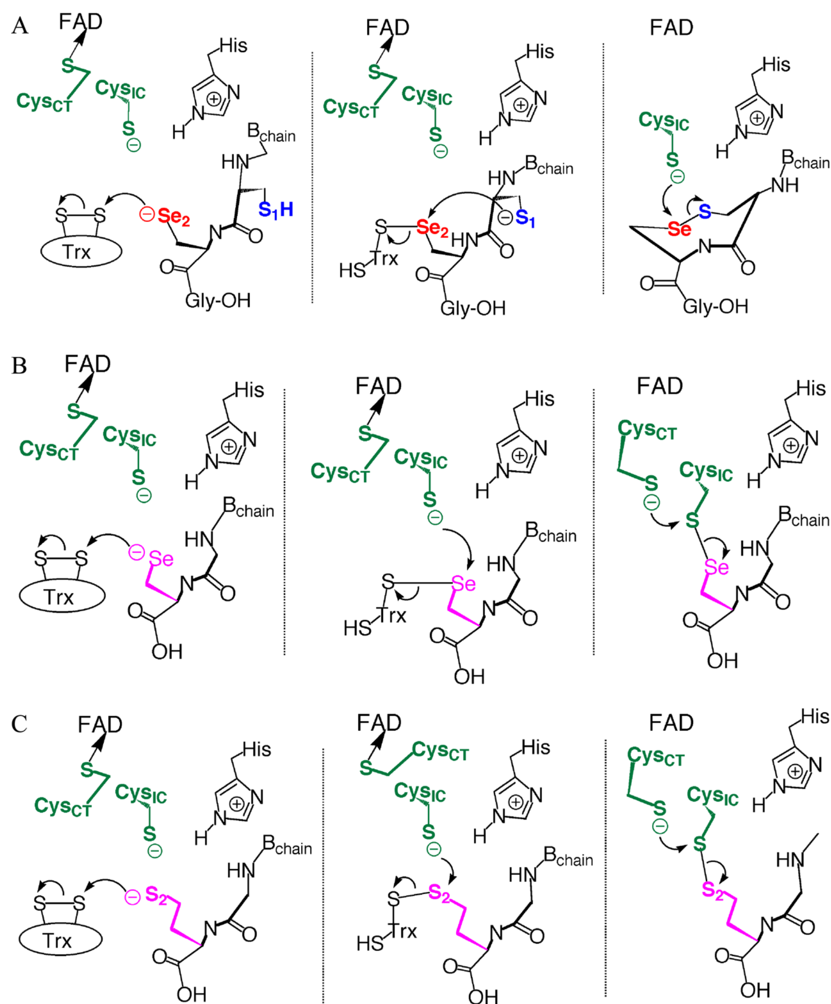


Figure 7. Partial mechanism for WT TR and mutant enzymes **7** and **8**. Because of space considerations, we begin our depiction of the mechanism with the EH_4 form of the enzyme and omit interactions between FAD and NADPH/$NADP^+$ as occurs in the enzyme. Please see ref 14 for a more detailed and complete description of the mechanism and electron flow from NADPH to the substrate. (A) The selenolate of the WT enzyme attacks the disulfide bond of Trx, forming a mixed selenosulfide bond between the enzyme and substrate (left). The mixed selenosulfide bond is then attacked by a resolving Cys residue, labeled as Cys_1 of the Cys_1-Sec_2 dyad (middle). The resolution step results in the formation of a unique eight-membered ring structure that must be "opened" for the catalytic cycle to begin again (right). Our data, based on mutant enzymes **7** and **8**, indicate that it is the Se atom of the ring that is attacked by the thiolate of Cys_{IC} (interchange Cys). (B) Enzyme **8** was able to reduce Trx at a rate higher than that of the Sec → Cys mutant (enzyme **2**) but lacks the resolving Cys residue (Cys_1) so that a ring formation pathway is impossible with this mutant. As shown above, the mechanism must bypass this ring-closing step and resolution of the mixed selenosulfide bond must be conducted by the thiolate of Cys_{IC}. (C) Mutant enzyme **7** contains only a single sulfhydryl group in the C-terminal redox center and, similar to enzyme **8**, can still reduce Trx. Mutant enzyme **7**, like enzyme **8**, must avoid a pathway that involves ring formation and instead rely on the thiolate of Cys_{IC} for the resolution step. Both of these results provide strong evidence that the residue in the second position of the C-terminal dyad of the mammalian enzyme (Sec) is responsible for both accepting and donating electrons. The Sec residue accepts electrons from the N-terminal redox center and then donates them to the substrate, Trx.

be a selenium atom of the Sec residue in the WT enzyme or the sulfur atom of the hCys residue in the mutant enzyme as shown in Figure 7. We note that neither enzyme **3** nor enzyme **6** has Trx-reductase activity.

■ CONCLUSION

This study has provided the first experimental evidence that the selenium atom is attacked in a "thiol/disulfide-like" exchange reaction in which Se replaces S.[48] Our data show that Se is responsible for both donating and accepting electrons during the catalytic cycle of mammalian TR and that the role of Se as an electron acceptor should be given consideration as a way in which Se helps to accelerate enzymatic reactions.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Biochemistry, University of Vermont, College of Medicine, 89 Beaumont Ave., Given Building Room B413, Burlington, VT 05405. E-mail: robert.hondal@uvm.edu. Telephone: (802) 656-8282. Fax: (802) 656-8220.

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 acknowledge the Vermont Cancer Center DNA Facility for sequencing services.

■ DEDICATION

This paper is dedicated to Professor Hans J. Reich (University of Wisconsin, Madison, WI) on the occasion of his retirement.

■ ABBREVIATIONS

Cys, cysteine; Cys_{CT}, charge-transfer Cys; Cys_{IC}, interchange cysteine; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gly, glycine; hCys, homocysteine; IPL, intein-mediated peptide ligation; M_r, molecular ratio; mTR3, mitochondrial TR from mouse; NADPH, reduced *β*-nicotinamide adenine dinucleotide phosphate; NMA, *N*-methylmercaptoacetamide; Sec, selenocysteine; Ser, serine; TB, Terrific Broth; TR, thioredoxin reductase; Tris, tris-(hydroxymethyl)aminomethane; Trx, thioredoxin; U, one-letter code for Sec; WT, wild-type.

■ ADDITIONAL NOTES

[a]The prime designation denotes amino acids that are part of the B-chain. The N- and C-terminal redox centers are on opposite subunits of the head-to-tail dimer.
[b]We note that there are large differences in the catalytic mechanisms of the cytosolic TR1 enzyme and the mitochondrial TR3 enzyme as reported in ref 47. As a result of these differences, the experiments performed here may not have been possible with TR1. Even so, results from the X-ray crystal structures of TR1 and TR3 show that attack at the selenium atom of the selenosulfide is the most likely path[22,23] as the data here show. All of our work on the TR reaction mechanism has been with the mitochondrial form of the enzyme, and only recently have we begun to appreciate differences in reaction mechanism between the two types. For example, the selenocystine-reductase activity of the Cys mutant of mTR3 is high,[6] while for the cytosolic enzyme, this activity is very low.[20]

■ REFERENCES

(1) Andersson, M., Holmgren, A., and Spyrou, G. (1996) NK-lysin, a disulfide-containing effector peptide of T-lymphocytes, is reduced and inactivated by human thioredoxin reductase. Implication for a protective mechanism against NK-lysin cytotoxicity. *J. Biol. Chem. 271*, 10116−10120.
(2) Bellisola, G., Fracasso, G., Ippoliti, R., Menestrina, G., Rosen, A., Solda, S., Udali, S., Tomazzolli, R., Tridente, G., and Colombatti, M. (2004) Reductive activation of ricin and ricin A-chain immunotoxins by protein disulfide isomerase and thioredoxin reductase. *Biochem. Pharmacol. 67*, 1721−1731.
(3) 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.
(4) Bjornstedt, M., Xue, J., Huang, W., Akesson, B., and Holmgren, A. (1994) The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. *J. Biol. Chem. 269*, 29382−29384.
(5) Kalantari, P., Narayan, V., Natarajan, S. K., Muralidhar, K., Gandhi, U. H., Vunta, H., Henderson, A. J., and Prabhu, K. S. (2008) Thioredoxin reductase-1 negatively regulates HIV-1 transactivating protein Tat-dependent transcription in human macrophages. *J. Biol. Chem. 283*, 33183−33190.
(6) 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.
(7) May, J. M., Cobb, C. E., Mendiratta, S., Hill, K. E., and Burk, R. F. (1998) Reduction of the ascorbyl free radical to ascorbate by thioredoxin reductase. *J. Biol. Chem. 273*, 23039−23045.
(8) Gromer, S., and Gross, J. H. (2002) Methylseleninate is a substrate rather than an inhibitor of mammalian thioredoxin reductase. Implications for the antitumor effects of selenium. *J. Biol. Chem. 277*, 9701−9706.
(9) Mustacich, D., and Powis, G. (2000) Thioredoxin reductase. *Biochem. J. 346* (Part 1), 1−8.
(10) Kumar, S., Bjornstedt, M., and Holmgren, A. (1992) Selenite is a substrate for calf thymus thioredoxin reductase and thioredoxin and elicits a large non-stoichiometric oxidation of NADPH in the presence of oxygen. *Eur. J. Biochem. 207*, 435−439.
(11) Hondal, R. J., and Ruggles, E. L. (2011) Differing views of the role of selenium in thioredoxin reductase. *Amino Acids 41*, 73−89.
(12) Arnér, E. S. (2010) Selenoproteins: What unique properties can arise with selenocysteine in place of cysteine? *Exp. Cell Res. 316*, 1296−1303.
(13) 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.
(14) 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.
(15) Lee, S. R., Bar-Noy, S., Kwon, J., Levine, R. L., Stadtman, T. C., and Rhee, S. G. (2000) Mammalian thioredoxin reductase: Oxidation of the C-terminal cysteine/selenocysteine active site forms a thioselenide, and replacement of selenium with sulfur markedly reduces catalytic activity. *Proc. Natl. Acad. Sci. U.S.A. 97*, 2521−2526.
(16) Mugesh, G., and Sing, H. B. (2000) Synthetic organoselenium compounds as antioxidants: Glutathione peroxidase activity. *Chem. Soc. Rev. 29*, 347−357.
(17) Pearson, R. G., and Songstad, J. (1967) Application of the principle of hard and soft acids and bases to organic chemistry. *J. Am. Chem. Soc. 89*, 1827−1836.
(18) Pearson, R. G., and Songstad, J. (1968) Nucleophilic reactivity constants toward methyl iodide and trans-$[Pt(py)_2Cl_2]$. *J. Am. Chem. Soc. 90*, 319−326.
(19) Eckenroth, B., Harris, K., Turanov, A. A., Gladyshev, V. N., Raines, R. T., and Hondal, R. J. (2006) Semisynthesis and characterization of mammalian thioredoxin reductase. *Biochemistry 45*, 5158−5170.
(20) 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.
(21) Fritz-Wolf, K., Kehr, S., Stumpf, M., Rahlfs, S., and Becker, K. (2011) Crystal structure of the human thioredoxin reductase-thioredoxin complex. *Nat. Commun. 2*, 383.
(22) 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.
(23) 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.
(24) 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.
(25) Lacey, B. M., Eckenroth, B. E., Flemer, S., and Hondal, R. J. (2008) Selenium in thioredoxin reductase: A mechanistic perspective. *Biochemistry 47*, 12810−12821.

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

(27) 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.

(28) Steinmann, D., Nauser, T., and Koppenol, W. H. (2010) Selenium and sulfur in exchange reactions: A comparative study. *J. Org. Chem. 75*, 6696−6699.

(29) 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.

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

(31) Gough, J. D., Williams, R. H., Jr., Donofrio, A. E., and Lees, W. J. (2002) Folding disulfide containing proteins faster with an aromatic thiol. *J. Am. Chem. Soc. 124*, 3885−3892.

(32) DeCollo, T. V., and Lees, W. J. (2001) Effects of aromatic thiols on reactions that occur during protein folding. *J. Org. Chem. 66*, 4244−4249.

(33) Basu, P., Nemykin, V. N., and Sengar, R. S. (2003) Synthesis, spectroscopy, and redox chemistry of encapsulated Oxo-Mo(V) centers: Implications for pyranopterin-containing molybdoenzymes. *Inorg. Chem. 42*, 7489−7501.

(34) Grice, R., and Owen, L. N. (1963) Cytotoxic compounds IV: Substituted benzyl halides. *J. Chem. Soc. 1963*, 1947−1954.

(35) Kawai, H., Sakamoto, F., Taguchi, M., Kitamura, M., Sotomura, M., and Tsukamoto, G. (1991) 2-Oxo-1,3-dioxoles as specific substrates for measurement of arylesterase activity. *Chem. Pharm. Bull. 39*, 1422−1425.

(36) 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.

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

(38) Gough, J. D., Gargano, J. M., Donofrio, A. E., and Lees, W. J. (2003) Aromatic thiol pK_a effects on the folding rate of a disulfide containing protein. *Biochemistry 42*, 11787−11797.

(39) Huber, R. E., and Criddle, R. S. (1967) Comparison of the chemical properties of selenocysteine and selenocystine and their sulfur analogs. *Arch. Biochem. Biophys. 122*, 164−173.

(40) Danehy, J. P., and Noel, C. J. (1960) The relative nucleophilic character of several mercaptans toward ethylene oxide. *J. Am. Chem. Soc. 82*, 2511−2515.

(41) Danehy, J. P., Elia, V. J., and Lavelle, C. J. (1971) Alkaline decomposition of organic disulfides. IV. Limitation on the use of Ellman's reagent. 2,2′-Dinitro-5,5′-dithiodibenzoic acid. *J. Org. Chem. 36*, 1003−1005.

(42) Kingery, D. A., and Strobel, S. A. (2012) Analysis of enzymatic transacylase Brønsted studies with application to the ribosome. *Acc. Chem. Res. 45*, 495−503.

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

(44) 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.

(45) Kim, H. Y., and Gladyshev, V. N. (2004) Methionine sulfoxide reduction in mammals: Characterization of methionine-*R*-sulfoxide reductases. *Mol. Biol. Cell 15*, 1055−1064.

(46) 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.

(47) Rackham, O., Shearwoo, A. M., Thyer, R., McNamara, E., Davies, S. M., Callus, B. A., Miranda-Vizuete, A., Berners-Price, S. J., Cheng, Q., Arner, E. S., and Filipovska, A. (2011) Substrate and inhibitor specificities differ between human cytosolic and mitochondrial thioredoxin reductases: Implications for development of specific inhibitors. *Free Radical Biol. Med. 50*, 689−699.

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