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

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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 Cys497-Sec498 dyad of the C-terminal redox center was mutated to either a Ser497-Cys498 dyad or a Cys497-Ser498 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 Ser497-Cys498 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 on 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-Seccoo 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 electrons 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
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, 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. 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-Sec dyad was mutated to a Ser-97-Cys-98 redox 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 (CysIC) and a charge-transfer Cys (CysCT) residue that interacts with the flavin coenzyme. As explained in the legend of Figure 1B, the thiolate of CysIC 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-Fe 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 CysIC 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-pKᵅ leaving group.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 pKᵅ 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 CysIC, 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.
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. 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 disulphides 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. Peptides were cleaved from the resin using trisfluoroacetic acid (TFA) containing triisopropylsilane and water in a 96:2:2 ratio. The cleavage volume was reduced by evaporation under a stream of N₂ 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/acetoni trile 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 mTR and the C-terminal tetrapeptide as GCUG. Thus, the WT enzyme is abbreviated as mTR3-GCUG throughout this report. Mutants are abbreviated as mTR3-aa1aa2aa3aa4 (using one-letter codes for the amino acids). The truncated mTR enzymes missing either the NMA or the chitin binding domain (CBD) 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. All enzyme constructs were purified using hydrophobic and anion exchange chromatography as described previously. 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 selenocysteine as substrates of TR have been previously described. 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 selenocysteine as the substrate contained 500 mM potassium phosphate (pH 7.0), 10 mM EDTA, 200 μM NADPH, and various concentrations of selenocysteine.

**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_s 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 disulphide 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

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.

advantage of the experimental design shown in Figure 2 is that it allows for the study of the kinetics of the thiol/disulphide exchange step that occurs between N- and C-terminal redox centers. We refer to this step simply as “the exchange step” hereafter.

The structure, pK_a values, and specific disulphide-reductase activities for these peptide substrates (I–IV) using mTRΔ8 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 disulphide-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 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. 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 disulphide 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 CysIC during the exchange step. Thus, the analysis of the Brønsted data supports the Se as an electrophile model mentioned in the

![Figure 3. Brønsted plot for the truncated enzyme with peptide disulphide substrates. The plot shows that over this range of pK_a values, high disulphide 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.](image)

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

| Peptide Number | Peptides I–IV | pK_a of S_2 | Activity at 1 mM peptide (mol NADPH/min/mol enzyme) | Relative Activity |
|----------------|---------------|-------------|--------------------------------------------------|-----------------|
| I              | X = SO_2H     | 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              |               | Se =−5.2^b  | 177                                               | 30.5            |
| VI             |               | −8.3^c      | 0.09                                              | 0.015           |
| VII            |               | −8.3^c      | 0.03                                              | 0.005           |
| VIII           |               | Se =−5.2^b  | 6.75                                              | 1.16            |
| DTNB           | HOOC−COOH     | 4.75^d      | 2000                                              | 345             |

^aTaken from ref 38. ^bTaken from ref 39. ^cTaken from ref 40. ^dTaken from ref 41. The data reported here for peptides V–VIII and DTNB was taken from ref 25.
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 pKₐ) with the slowest rate and peptide I (lowest pKₐ) 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 pKₐ 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 S₂ 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 S₁–S₂ bond is polarized because of the presence of the para-substituted aryl group and would be further polarized if S₁ were near a positive charge in the active site, such as His463⁺,⁴ which acts as an acid/base catalyst during the enzymatic reaction cycle. Both of these factors would help to make S₂ electron deficient (make S₂ 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 S₁ 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 be explained if the S₁–S₂ 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.⁸ ⁴⁵ A reasonable conclusion from these data is that Se confers polarization to a S–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 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 Cys₄₆₃ by positioning atom S₁ 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 S₂ of the disulfide of IV and the carboxyate 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 carboxyate 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.
that differ by only a single feature. For example, the V:VI turnover ratio shows that bond polarization contributes a factor of $\sim2000$ 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.

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. 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 disulfide bond.

**Functional Assay for Assessing Peptide Incorporation in IPL.** Enzymes 3–8 were constructed by using IPL. 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. IPL usually involves ligation of a peptide containing an N-terminal Cys, Sec, or hCys residue to a thioester-tagged protein. 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
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. 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 Cys1-Sec2 redox dyad and selenium acted as the acceptor of electrons from the N-terminal redox center. 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-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 CysIC 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 Cys1-Sec2 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 χα 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 CysIC 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...
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. 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.

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*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 EH4 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 Cys₈ of the Cys₈-Sec₂ 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 Cys₈ (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 (Cys₁) 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 Cys₈. (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 Cys₈ 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.
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■ ACKNOWLEDGMENTS

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■ 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(CP), charge-transfer Cys; Cys(SO), interchange cysteine; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gly, glycine; hCys, homocysteine; IPER, intein-mediated peptide ligation; Mr, 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

“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.

“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 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 selenocysteine-reductase activity of the Cys mutant of mTR3 is high, while for the cytosolic enzyme, this activity is very low.

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