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Characterization of Mitochondrial Thioredoxin Reductase from *C. elegans*

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Abstract

Thioredoxin reductase catalyzes the NADPH dependent reduction of the catalytic disulfide bond of thioredoxin. In mammals and other higher eukaryotes, thioredoxin reductases contain the rare amino acid selenocysteine at the active site. The mitochondrial enzyme from *Caenorhabditis elegans* however, contains a cysteine residue in place of selenocysteine. The mitochondrial *Caenorhabditis elegans* thioredoxin reductase was cloned from an expressed sequence tag and then produced in *Escherichia coli* as an intein-fusion protein. The purified recombinant enzyme has a k_{cat} of 610 min⁻¹ and a K_m of 610 μ M using *Escherichia coli* thioredoxin as substrate. The reported k_{cat} is 25% of the k_{cat} of the mammalian enzyme and is 43 fold higher than a cysteine mutant of mammalian thioredoxin reductase. The enzyme would reduce selenocystine, but not hydrogen peroxide or insulin. The flanking glycine residues of the GCCG motif were mutated to serine. The mutants improved substrate binding, but decreased the catalytic rate.

Keywords

selenocysteine; *C. elegans*; mitochondrial; thioredoxin reductase; intein

Introduction

Thioredoxin reductase (TR) is a member of the family of pyridine nucleotide-disulfide reductases, which function to maintain cellular redox homeostasis [1]. TR is an essential part of the thioredoxin system composed of TR, thioredoxin (Trx), and NADPH [2]. Members of this family accept reducing equivalents, usually in the form of NADPH, which donates a hydride to an enzyme bound cofactor (FAD), which in turn forms a charge-transfer complex with an enzyme disulfide and then transfers the electrons to the target substrate. High molecular weight TR's contain an additional C-terminal redox center. This C-terminal redox center contains a dithiol/disulfide redox pair, which first accepts two electrons from the N-terminal dithiol/disulfide, and it is this C-terminal motif that catalyzes reduction of the target substrate [3].

In many eukaryotic TR's the C-terminal redox center contains the rare, 21st amino acid selenocysteine (Sec, U) as part of a conserved tetrapeptide GCUG motif [4-5]. The Sec residue of this motif has been shown to be essential for function and this residue is thought to impart the enzyme with broad substrate specificity including hydrogen peroxidase activity [6].

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However, not all TR's contain Sec and are able to catalyze the reduction of the catalytic disulfide bond of Trx with a cysteine (Cys) residue. For example, in the case of the TR from *Drosophila melanogaster* (*D.m.* TR), the enzyme contains a C-terminal SCCS motif and this enzyme can reduce its cognate Trx with high efficiency, thus demonstrating that the reaction can be catalyzed without the need of a selenium atom [7]. It has recently been proposed that the reason that the *D.m.* TR can function with high catalytic efficiency using Cys in place of Sec, is that the flanking serine residues increase the nucleophilicity of the thiol nucleophile by mediating proton transfer from the attacking thiol to the leaving group sulfur atom of the substrate [8]. Mutation of the N-terminal serine residue to a glycine residue had little effect on catalytic parameters, while mutation of the C-terminal serine to a glycine residue decreased k_{cat} by two thirds. Thus it seems that for the *D.m.* TR, the C-terminal serine residue plays some role in catalysis.

However, not all TR's in which the Sec residue has been replaced with Cys contain flanking serine residues. The TR from *Anopheles gambiae* (*A.g.* TR) contains an N-terminal threonine, and in the case of the mitochondrial TR from *C. elegans* (*C.e.* TR3) the flanking residues are glycine [9-10].

It has been previously demonstrated that *C. elegans* contains two TR's, a cytosolic form, containing Sec with a C-terminal GCUG tetrapeptide motif, and a mitochondrial form containing Cys with a C-terminal GCCG motif [10]. Curiously, *C. elegans* has acquired the machinery necessary to recode a UGA stop codon as a sense codon for selenocysteine for a single enzyme, and some properties of this enzyme have been previously reported [11].

Our interest in this paper is in the mitochondrial TR from *C. elegans* with the naturally occurring GCCG tetrapeptide redox center. This enzyme has not been previously cloned, nor has its catalytic properties been reported. In light of the proposal by Arnér and coworkers regarding the importance of the flanking serine residues for cysteine-containing TR's, we wished to characterize the catalytic properties of *C.e.* TR3 in order to ascertain the importance of the flanking residues of this tetrapeptide motif to the importance of enzymatic catalysis.

In this paper, we report on the cloning, recombinant production, purification, and enzymatic characterization of *C.e.* TR3.

Materials And Methods

Materials

All reagents were purchased from either Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA). Restriction endonucleases Nde I, Sap I, Hind III, Eco RI, Vent DNA Polymerase, T4 DNA ligase, plasmid pTYB1, chitin-agarose, and reaction buffers were supplied by New England Biolabs (Beverly, MA). Recombinant *E. coli* thioredoxin was purchased from American Diagnostica (Greenwich, CT). Enzyme kinetics were performed on a Cary50 UV/Vis spectrophotometer. DNA oligonucleotides for cloning and mutagenesis were purchased from IDT, Inc. (Coraleville, IA).

Mutagenesis and Transformation

The coding sequence for *C.e.* TR3 was amplified from an EST clone purchased from Open Biosystems (clone ID ZK637.10) using PCR. The upstream primer had the sequence 5'-AACAGACAT ATGTTCTCATCAAATAAATTTGATCTGATTG-3', and the downstream primer was of sequence 5'-ACAGCCGCTCTTCAGCATCCACAGCAT CCCTGAGTT-3'. The PCR was performed on a Perkin Elmer GeneAmp PCR System 2400. The PCR reaction (100 μ L) contained 100 pg of template DNA (pDONR201, containing the coding sequence),

50 pmol of each primer, 2 units of Vent DNA polymerase, 200 μ M of each dNTP, in a buffer containing 20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , and 0.1% Triton X-100, pH 8.8. The steps in the PCR reaction were as follows: 96 °C (initial denaturation) for two minutes followed by 25 cycles of 96 °C (45 seconds), 55 °C, (30 seconds), and 72 °C (3 minutes). The amplification reaction was monitored by analytical agarose gel electrophoresis (staining with ethidium bromide). The DNA from the PCR reaction was then purified using the Qiagen QIAquick PCR Purification Kit. The PCR product was then treated with restriction enzymes Nde I and Sap I for 2 h at 37 °C in 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT pH 7.9 in a total volume of 50 μ L. The digest was then purified using the Qiagen QIAquick PCR Purification Kit and the purified DNA was used in a ligation reaction to subclone the amplified gene into plasmid pTYB1. 200 ng of plasmid pTYB1 and 400 ng of PCR product were incubated with 400 units of T4 DNA ligase at 16 °C overnight. The ligase was inactivated by heating the reaction at 70 °C for 30 min. The ligation reaction was then incubated at 37 °C with Eco RI for 2 h to enhance the amount of positive clones because Eco RI will only cut plasmids that have not been ligated with the PCR insert. The resulting plasmid (pCETR3) encodes a fusion protein consisting of *C.e.* TR3, VMA1 intein, and a chitin-binding domain.

E. coli DH5 α cells made competent by CaCl_2 , were transformed with 20 ng of pCETR3 [12]. The cells were plated on LB-agar containing ampicillin (200 μ g/mL). Positive clones were screened by restriction analysis using Hind III and Nde I and analyzed by analytical agarose gel electrophoresis. Several positive clones were then selected for DNA sequencing to verify the wild-type coding sequence. All sequencing was performed by the University of Vermont DNA Sequencing Facility using an ABI 3100-Avant Genetic Analyzer.

Two mutant *C.e.* TR's were also constructed using PCR using the same conditions listed above except that downstream primer 5' - ACAGCCGCTCTTCAGCATGAACAGCATCCCTGA GTTCTT-3' was used to create a mutant with C-terminal sequence GCCS (abbreviated as TR-GCCS) and downstream primer 5' -ACAGCCGCTCTTCAGCATGAACAGCATGACTGAGTT CTT-3' to create a mutant with C-terminal sequence of SCCS (abbreviated as TR-SCCS).

Protein Production

E. coli ER2566 cells were used for production of recombinant wild-type (WT) and mutant *C.e.* TR3 enzymes. The cells were transformed with plasmid pCETR3 and plated on LB-ampicillin plates containing 200 μ g/mL ampicillin and incubated at 37 °C overnight. A single colony isolated from the LB/ampicillin plate was used to grow a 100 mL inoculum culture of LB (200 μ g/mL ampicillin). This culture was incubated overnight at 37 °C with shaking. 10 mL of inoculum culture was added to each of 6 $\times$ 1L baffled Pyrex Fernbach flasks of TB media containing 200 μ g/mL ampicillin. The cells were incubated at 37 °C with shaking (100 r.p.m.) until the O.D._{600nm} reached 1.0. The cells were then chilled on ice until the temperature decreases to 20 °C and induced by adding IPTG to a final concentration of 0.5 mM followed by incubation at 20 °C overnight with shaking (100 r.p.m.) The cells were harvested by centrifugation in a Beckman J21B centrifuge (JA-14 rotor) at 7000 $\times g$ at 4 °C for 10 min and then stored at -20 °C.

Enzyme Purification

The frozen cell pellets were thawed on ice and resuspended in 200 mL of 50 mM MOPS, 150 mM NaCl, pH 7.0 until homogeneous. The homogenate was sonicated with a Branson 350 Sonifier (Danbury, CT) on ice to break open the cells. The sample was then centrifuged with a Beckman J21B centrifuge (JA-14 rotor) at 7000 $\times g$ for 90 minutes at 4 °C. The

supernatant was gravity loaded on to a 60 mL column of chitin-agarose with a flow rate of 0.5-1.0 mL/min. The column was washed with 50 mM MOPS, 150 mM NaCl, pH 7.0 until the absorbance at 280 nm reached 0.05. This was followed by a 1 L wash of 50 mM MOPS, 500 mM NaCl pH 7.0. As a final step, the column was then washed with 1 volume of column buffer that contained 140 mM β ME (Since the fusion protein was resistant to cleavage by β ME, we found that a wash with this thiol greatly improved the purity of the protein in this step). The enzyme was then liberated from the intein-fusion protein by incubation of the chitin resin with column buffer (50 mM MOPS, 500 mM NaCl pH 8.0) containing a small molecule thiol. The thiol-cleavage fractions were concentrated by ultrafiltration using an Amicon Ultra 30000 MWCO (Millipore) concentrator to a final volume of 1-2 mL. gravity loaded onto a gel filtration column (S200, Pharmacia) of dimensions 3.8 cm $\times$ 97 cm. The gel filtration column was equilibrated with 50 mM MOPS, 500 mM NaCl pH 7.0. 3.0 mL fractions were collected and the fractions were analyzed by UV/Vis spectrophotometry at 280 nm and 460 nm. Peak fractions were assayed for DTNB reductase activity and analyzed by SDS-PAGE (12% gels). Peak fractions exhibiting DTNB reductase activity were pooled and concentrated by ultrafiltration. The protein concentration was determined using an extinction coefficient of 22.6 mM⁻¹·cm⁻¹ at 460 nm. The concentrated protein was then stored at 4 °C until needed. The optimal thiol for cleavage was determined by a cleavage assay as described in the next section.

Optimization of Thiol-Mediated Cleavage

Initially, we found the fusion protein to be resistant to cleavage using 70 mM β ME as a thiol at pH values ranging from 7-8. In order to find the best conditions for thiol-mediated cleavage, we explored a variety of thiols and pH values to liberate our target protein (*C.e.* TR3) from the chitin-agarose. Four different batches of chitin resin (6 mL slurry) were treated with 15 mL of buffer (50 mM MOPS, 500 mM NaCl, pH 7.0) containing either 140 mM beta-mercaptoethanol (β ME), 140 mM *N*-methylmercaptoacetamide (NMA), 140 mM dithiothreitol (DTT), or 140 mM mercaptoethanesulfonic acid (sodium salt) (MESNA), and incubated at room temperature overnight while mixing using a platform rocker (the pH of the slurry was adjusted to 7.0 after the addition of thiol). A control reaction was done in which buffer was added to the slurry that did not contain thiol. The resin was then filtered and then washed with column buffer that did not contain thiol. The combined effluent was concentrated by ultrafiltration and the samples were then analyzed by 12% SDS-PAGE electrophoresis.

Enzyme activity assays

The activity of the recombinant proteins were tested for their ability to reduce DTNB (5, 5'-dithiobis(2-nitrobenzoate) by monitoring the increase in absorbance at 412 nm [13]. The assay contained 4 nM enzyme, 100 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA, 0.2 mM NADPH, and DTNB (0 – 5 mM) in an assay volume of 0.5 mL. Thioredoxin reductase activity was measured using *E. coli* Trx as substrate, by monitoring the consumption of NADPH by measuring the decrease in absorbance at 340 nm as previously described [14]. Briefly, assay mixtures contained 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.15 mM NADPH, 0.16 mM bovine insulin, and *E. coli* Trx (0 – 420 mM) in a volume of 0.5 mL. The TR concentration was 40 nM for this assay. The data was fit to a straight line over a 2 min range on the Varian-Cary 50 spectrophotometer to calculate the change in absorbance per min.

Activity Towards Other Substrates

WT enzyme was tested for its ability to reduce selenocystine, hydrogen peroxide and bovine pancreatic insulin. These assays were carried out as previously described with slight modification [14]. The assay conditions were: 100 mM potassium phosphate, pH 7.0, 10

mM EDTA, 0.2 mM NADPH, 200 nM WT enzyme, in a final volume of 0.5 mL for assays using selenocystine or H₂O₂ as substrates. The assay for bovine pancreatic insulin was tested under the following conditions: 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.15 mM NADPH, and 400 nM WT enzyme in a 0.5 mL assay. Activity was measured by following the decrease in absorbance at 340 nm, indicating consumption of NADPH. The concentration of selenocystine in the assay was 85 μ M. Hydrogen peroxidase activity was measured using concentrations of H₂O₂ varying from 0 to 500 μ M. Insulin concentrations were in the range of 0.2 to 1 mM.

pH Rate Profiles

WT and mutant enzymes were tested for their ability to reduce *E. coli* Trx at varying pH. The assay was similar to the assay described above with slight modification. In brief, the assay buffer contained 200 mM each of sodium acetate, sodium phosphate (monobasic), sodium phosphate (dibasic), and boric acid. The pH of the buffer was adjusted by 0.5 pH units with HCl or NaOH to give the desired pH value. Insulin was omitted due to precipitation at low pH and each assay reaction contained 500 μ M *E. coli* Trx, 1 mM EDTA, 0.15 mM NADPH, and 60 nM enzyme in a final volume of 0.5 mL.

Results And Discussion

Cloning and Production of Recombinant Enzymes in *E. coli*

Sequence analysis of the amplified PCR product indicated that the cloned DNA was identical to that of Genbank accession # Z11115, which was previously identified as the mitochondrial TR from *C. elegans* [10]. For production as a recombinant protein in *E. coli*, we removed the mitochondrial signaling sequence (MLLSTFKRHLPIRRL) and fused the enzyme to an intein-chitin binding domain that allows for affinity purification. Figure 1 details our strategy for isolating the recombinant enzyme. The cell extract is loaded onto a chitin-agarose column and after extensive washing with column buffer, the *C.e.* TR3 enzyme can be liberated by thiol-mediated cleavage. The liberated protein will elute from the column with its C-terminus in the form of a thioester. This thioester group will hydrolyze over several days to produce the free carboxylate, typical of C-termini of proteins [15]. The chitin-agarose column affords a high level of purification of the enzyme as shown by the 12% SDS-PAGE gel in Figure 2A. The apparent MW of *C.e.* TR3 is ~55 kDa, typical of high molecular weight thioredoxin reductases. [3]. The enzyme is then purified to greater than 95% homogeneity (judged by SDS-PAGE) after S200 size-exclusion chromatography (data not shown). The intein-fusion strategy allows us to purify the enzyme in a two-step process, and also allows us to take advantage of the protein engineering utility of inteins for future experiments [16]. The intein strategy has proved useful to us in the past, however in the case of *C.e.* TR3, this strategy proved to be challenging as cleavage from the intein through the use of a thiol proved difficult. Figure 2B shows the cleavage of mouse TR3 from an intein fusion protein using different thiols. The thiols β ME, NMA, and DTT all cleaved with similar efficiency, while MESNA cleaved with the least efficiency. Initially we tried to cleave the *C.e.* TR3-intein fusion protein with β ME, which resulted in little or no cleavage. The results of our thiol cleavage assay in Figure 2A demonstrate the superiority of NMA as a cleavage thiol. DTT and β ME did not cleave the construct to any significant extent at either pH 7.0 or pH 8.0. MESNA (data not shown) also demonstrated an inability to cleave the fusion protein. However, as can be seen in Figure 2A, NMA cleaved the fusion protein to a significant extent at pH 7.0 and allowed us to use the intein strategy as a method for isolating the recombinant enzyme with a final yield of 2 mg/L of cell culture. The purified WT and mutant enzymes absorbance spectra were scanned from 200 to 600 nm (data not shown), with characteristic maxima at 370 nm and 460 nm for the flavin-bound cofactor present.

Thioredoxin Reductase Activity

The thioredoxin reductase activity of *C.e.* TR3 was determined using *E. coli* Trx as substrate. *E. coli* Trx has been used as a standard to measure the thioredoxin reductase activity of many different types of TR's, however it has been shown that TR's from different species prefer their cognate thioredoxin as a substrate, and the preference for the cognate thioredoxin molecule can vary greatly between species [7].

This activity was measured using a well-developed assay as described by Holmgren [14]. The results of the assay containing NADPH, bovine insulin, and *E. coli* Trx in 50 mM potassium phosphate buffer for WT and mutant *C.e.* TR's are shown in Figure 3 as a Michaelis-Menten plot and the kinetic data is summarized in Table 1. The results show that the WT enzyme has high activity towards *E. coli* Trx as measured by its high value of k_{cat} (610 min^{-1}). However, the WT enzyme also has a high K_{m} value ($610 \mu\text{M}$) towards *E. coli* Trx, indicating that the non-cognate Trx is not the optimal substrate for the enzyme. It is likely that the *C.e.* TR3 would bind its cognate Trx with much higher affinity resulting in a lower K_{m} value. This result is similar to the results of the TR from *Plasmodium falciparum* (*P.f.*), which also has high activity towards *E. coli* Trx, but also displays a high K_{m} value [17]. When the *P.f.* TR is assayed using its own cognate Trx, the k_{cat} value is similar to the k_{cat} value when *E. coli* Trx is the substrate, but the K_{m} value for the cognate Trx is much lower (Table 2). The high value of k_{cat} towards *E. coli* Trx does indicate that like the TR's from *Drosophila melanogaster* and *Plasmodium falciparum*, the reduction of the catalytic disulfide bond of Trx does not require selenium, and furthermore that the enzymes containing an active-site thiolate nucleophile are capable of achieving catalytic rates comparable to the mammalian enzymes which contain Sec.

The WT enzyme has unusual kinetic properties at low substrate concentration ($0 - 30 \mu\text{M}$). When measuring the absorbance at 340 nm during the assay, the absorbance should decrease indicating consumption of NADPH and turnover of substrate. However at low substrate concentration, the absorbance was observed to increase. This either indicates that the enzyme has NADPH oxidase activity at low substrate concentration, or the enzyme exhibits cooperativity. Alternatively, this result may be an artifact of using a non-cognate Trx as substrate. Neither mutant enzyme displayed this property.

The mitochondrial TR from *C. elegans* (*C.e.* TR3) is of interest because of the proposal by Arnér and coworkers that *D.m.* TR can achieve high catalytic activity without the need for Sec because of the presence of flanking serine residues in the C-terminal tetrapeptide SCCS redox center of the enzyme [8]. These serine residues take the place of flanking glycine residues in the mammalian C-terminal tetrapeptide motif of GCUG. It was proposed that the serine residues act as general bases with the assistance of a nearby histidine residue to deprotonate the Cys residues during the catalytic cycle of the enzyme. *C.e.* TR3 is a naturally occurring Cys-containing TR (Cys replaces Sec in the active site) that has a flanking glycine residue on each side of the vicinal Cys motif. Thus in the case of *C.e.* TR3, there is no requirement for flanking serine residues to assist in proton abstraction and the enzyme can achieve high activity in the absence of Sec. The mechanism of *D.m.* TR may require the presence of flanking serine residues to achieve catalysis, but this is certainly not the case for the *C.e.* TR3.

We have also constructed several mutants to further explore the hypothesis put forth by Arnér and coworkers that flanking serine residues are essential for TR's with vicinal Cys residues [8]. In *D.m.* TR, it was shown that the C-terminal serine was more important to catalysis since mutation of this residue to glycine affected the catalytic activity the most. When we changed the C-terminal glycine residue of *C.e.* TR3 to serine we did not observe an increase in activity as might be expected if the presence of a serine in this motif could

function to deprotonate the C-terminal Cys residue as in the Arnér proposal. In fact, the activity of the enzyme decreased 2-fold in k_{cat} , however, this mutant did show better binding of the substrate as K_{m} decreased nearly 9-fold. Thus this residue may be important for substrate binding and further differences in K_{m} may be observed with this mutant when comparing *E. coli* Trx to the cognate Trx from *C. elegans*. However, the mitochondrial Trx from *C. elegans* has not been cloned and as far as we know is unavailable. This is an area for future work. When both flanking glycine residues are mutated to serine (the opposite experiment to that in ref. [8]), the activity is 2.5 fold lower in k_{cat} and has a 3-fold lower K_{m} . The results show that similar to the proposal of Arnér and coworkers, the C-terminal flanking residue seems to be more important for catalysis than the N-terminal flanking residue in *C.e.* TR3.

DTNB reductase activity

DTNB has been widely used as a substrate for thioredoxin reductases as the disulfide bond of this substrate is highly reactive and this provides a convenient method for assaying the enzyme during purification. The catalytic constants using DTNB are usually reported as a means of comparing the activity of different TR's towards a common substrate [6]. The Michaelis-Menten plot for *C.e.* TR3 using DTNB as a substrate is shown in Figure 4. The enzyme has a k_{cat} of 134 min^{-1} and a K_{m} of 0.42 mM. This compares to a k_{cat} of $2,100 \text{ min}^{-1}$ and a K_{m} of 100 mM for the mammalian Sec-containing enzyme [18]. The k_{cat} of the *C.e.* TR3 for DTNB is similar to that of the mammalian U498C mutant (Sec to Cys mutation) (220 s^{-1}) [18]. Sometimes a specific activity for DTNB at a concentration of 5 mM is reported in the literature [6]. For example, calf liver TR has a specific activity of 1,333 mol NADPH/min/mol TR at 5 mM DTNB, while the corresponding value for *C.e.* TR3 is 130. This most likely reflects the poorer nucleophilicity of the active site thiolate of *C.e.* TR3 compared to the selenolate for the mammalian enzyme.

C.e. TR3 was also tested for its ability to reduce several other substrates (Table 3). The enzyme showed no detectable hydrogen peroxidase activity and no ability to reduce disulfide bonds of peptides such as in insulin. The lack of hydrogen peroxidase activity is consistent with previous reports of the requirement for selenium to catalyze this reaction and reflects the poorer nucleophilicity towards peroxide bonds by sulfur in comparison to selenium [20]. The enzyme was able to utilize selenocystine as a substrate and we report a specific activity of 29 min^{-1} using $85 \mu\text{M}$ selenocystine.

pH Rate Profile

WT and mutant enzymes were tested for their ability to reduce *E. coli* Trx at varying pH. The WT enzyme was found to have a pH optimum centered near pH 8.0 (Figure 5). Mutant enzymes containing altered C-terminal tetrapeptides (GCCS and SCCS) also showed a pH optimum near 8.0, but the mutant profiles are somewhat broader than the WT enzyme. The mammalian Sec-containing enzyme shows a pH optimum near 7.0, while the pH optimum is shifted between 8-10 for the mammalian U498C mutant [6]. The *C.e.* TR3 enzyme, like the mammalian U498C mutant also shows a pH optimum shifted towards 8.0, but the profile is narrower compared to the Cys-containing mammalian mutant. *C.e.* TR3 likely evolved to potentiate the nucleophilicity of an active-site thiolate, potentially through the use of a general base.

The mitochondrial TR of the nematode *C. elegans* has evolved down a different path than that of the mammalian mitochondrial enzyme. While the cytosolic *C. elegans* TR1 contains Sec, its mitochondrial counterpart does not. It is not known why *C. elegans* has evolved to contain the entire Sec decoding apparatus for one Sec-containing enzyme [11]. One possibility for the mitochondrial *C.e.* TR is that it originally contained Sec, but was lost

through evolution and was replaced with Cys. It has been proposed that the occurrence of Sec in proteins is the result of a recent evolutionary event [21] and that the presence of Sec in TR enables the enzyme to have additional biological roles inside the cell other than the reduction of Trx [22]. Thus, the function of the mitochondrial TR from *C. elegans* may be limited to the reducing the catalytic disulfide bond of Trx because the redox-sensing function of TR may be performed by other proteins in this organelle of *C. elegans*.

In conclusion, the *C.e.* TR3 enzyme containing Cys in place of Sec has high catalytic activity towards *E. coli* Trx, as has been observed for the Cys-containing TR of *D. melanogaster* [7]. The *C.e.* TR3 enzyme must use a mechanism that is slightly different from that of the *D.m.* TR since no requirement for flanking serine residues is observed.

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Figure 1. Fusion protein mechanism for affinity purification

The target protein, *C.e.* TR, was fused at its carboxy terminus with an engineered intein. The construct also has a chitin-binding domain (CBD) at the C-terminus, which allows for affinity purification with chitin-agarose. The CBD is a small peptide that has high affinity for chitin-agarose, and this resin provides a high level of purification of the fusion protein. The cysteine residue (of the intein) at the TR-intein junction, undergoes a N-S acyl shift resulting in the formation of a thioester bond at the TR-intein junction. Addition of a small thiol (NMA) mediates cleavage of TR from the intein, which remains bound to the chitin resin through the CBD. The liberated thioester tagged protein then undergoes hydrolysis yielding a native carboxy terminus.

Figure 2. Thiol mediated cleavage efficiency of different small molecule thiols

In (A), *C.e.* TR3 is cleaved with 140 mM thiol at pH 7.0. Lane 1: MWM. Lane 2: Fresh chitin resin without protein that has been treated with a 1% SDS solution as a control. Lane 3: The column wash (buffer) after fresh chitin resin has been loaded with protein extract. Lane 4: 1% SDS wash from a chitin column loaded with protein extract. Lane 5: β ME wash of chitin column. Lane 6: 1% SDS wash of column following β ME wash. Lane 7: DTT wash of chitin column. Lane 8: 1% SDS wash of column following DTT wash. Lane 9: NMA wash of chitin column. Lane 10: 1% SDS wash of column following NMA wash. The liberated *C.e.* TR3 is indicated by the arrow showing the band with an apparent MW of 55 kDa. In (B), mouse TR3 is produced as an intein fusion and cleaved with different thiols (50 mM) at pH 7.0. Lane 1: β ME. Lane 2: NMA. Lane 3: DTT. Lane 4: MESNA. In (B) NMA, DTT, and β ME all cleave with comparable efficiencies, with MESNA being the least efficient cleavage thiol. In (A) the superiority of NMA as a cleavage thiol is evident. In (C), the structures of each thiol, along with the corresponding pK_a values are shown [19].

Figure 3. Michaelis-Menten plot of WT and mutant enzymes using thioredoxin as substrate
Purified WT and mutant *C. elegans* TR's were assayed for their ability to reduce *E. coli* Trx. The activity was monitored by consumption of NADPH *via* a decrease in absorbance at 340 nm. Reaction mixtures contained 50 mM potassium phosphate pH 7.0, 1 mM EDTA, 150 μM NADPH, 160 μM bovine pancreatic insulin, 1-450 μM *E. coli* Trx and 60 nM enzyme. The WT enzyme is represented by the closed circles (TR-GCCG), open squares represent TR-GCCS, and closed diamonds represent TR-SCCS.

Figure 4. Michaelis-Menten plot of WT *C.e.* TR3 using DTNB as substrate

The assay contained 100 mM potassium phosphate, pH 7.0, 10 mM EDTA, 200 μ M NADPH, 4 nM enzyme, and 0-5.0 mM DTNB. Reactions were monitored over 2 min for an increase in absorbance at 412 nm indicating reduction of DTNB. Activity is expressed as mol of NADPH consumed/min/mol TR.

Figure 5. Activities of WT and mutant *C.e.* thioredoxin reductases as a function of pH

The thioredoxin reductase activity of WT and mutant enzymes was measured in buffers of varying pH. The activity was measured in 0.5 pH unit increments and is expressed as a percent of the maximal activity. Reaction mixtures contained 200 mM each of potassium phosphate (monobasic), potassium phosphate (dibasic), sodium acetate, and boric acid along with 1 mM EDTA, 150 μ M NADPH, 60 nM enzyme, and 500 μ M *E. coli* Trx. The WT enzyme is represented by the closed circles (TR-GCCG), open squares represent TR-GCCS, and closed diamonds represent TR-SCCS.

Table 1Kinetic Constants for WT and Mutant Enzymes using *E. coli* Trx as Substrate.

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\cdot\text{M}^{-1}$)
<i>C.e.</i> TR-GCCG (WT)	610 ± 36	610 ± 60	1×10^6
<i>C.e.</i> TR-GCCS	77 ± 3	318 ± 25	4.13×10^6
<i>C.e.</i> TR-SCCS	219 ± 14	242 ± 33	1.1×10^6

Table 2

Comparison of Kinetic Constants of TR's from Various Species.

Enzyme	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\cdot\text{M}^{-1}$)
rTR-GCUG ^a	<i>H. sapiens</i> Trx	3.3	2500	7.58×10^8
rTR-GCCG ^a	<i>H. sapiens</i> Trx	0.4	14.3	3.58×10^7
<i>C.e.</i> TR-GCCG	<i>E. coli</i> Trx	610	610	1×10^6
<i>P.f.</i> TR-GCGGGKCG ^b	<i>P.f.</i> Trx	2.1	1674	7.97×10^8
<i>P.f.</i> TR-GCGGGKCG ^b	<i>E. coli</i> Trx	500	1688	3.38×10^6
<i>D. m.</i> TR-SCCS ^c	<i>D.m.</i> Trx	7.1	1320	1.86×10^8
<i>A.g</i> TR-TCCS ^d	<i>A.g.</i> Trx	8.5	924	1.13×10^8

^aTaken from ref. [6].^bTaken from ref. [17].^cTaken from ref. [7].^dTaken from ref. [9].

Table 3Activity of *C. elegans* TR3 vs. Various Substrates.

Substrate	Activity (mol NADPH/min/mol of TR)
DTNB	130
Selenocystine (85 μ M)	29
Hydrogen peroxide	No activity
Insulin	No activity