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Roles of N-Terminal Active Cysteines and C-Terminal Cysteine-Selenocysteine in the Catalytic Mechanism of Mammalian Thioredoxin Reductase[1]

Noriko Fujiwara,[*] Tsuneko Fujii,[*] Junichi Fujii,[†] and Naoyuki Taniguchi[*,2]

[*]Department of Biochemistry, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871; and [†]Department of Biochemistry, Yamagata University School of Medicine, 2-2-2 Iidanishi, Yamagata, Yamagata 990-9585

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Mammalian thioredoxin reductase [EC 1.6.4.5], a homodimeric flavoprotein, has a marked similarity to glutathione reductase. The two cysteines in the N-terminal FAD domain (-Cys59-x-x-x-x-Cys64-) and histidine (His472) are conserved between them at corresponding positions, but the mammalian thioredoxin reductase contains a C-terminal extension of selenocysteine (Sec or U) at the penultimate position and a preceding cysteine (-Gly-Cys497-Sec498-Gly). Introduction of mutations into the cloned rat thioredoxin reductase gene revealed that residues Cys59, Cys64, His472, Cys497, and Sec498, as well as the sequence of Cys497 and Sec498 were essential for thioredoxin-reducing activity. To analyze the catalytic mechanism of the mammalian thioredoxin reductase, the wild-type, U498C, U498S, C59S, and C64S were overproduced in a baculovirus/insect cell system and purified. The wild-type thioredoxin reductase produced in this system, designated as WT, was found to lack the Sec residue and to terminate at Cys497. A Sec-containing thioredoxin reductase, which was purified from COS-1 cells transfected with the wild-type cDNA, was designated as SecWT and was used as an authentic enzyme. Among mutant enzymes, only U498C retained a slight thioredoxin-reducing activity at about three orders magnitude lower than SecWT. WT, U498C, and U498S showed some 5,5′-dithiobis(2-nitrobenzoic acid)–reducing activity and transhydrogenase activity, and C59S and C64S had substantially no such activities. These data and spectral analyses of these enzymes suggest that Cys59 and Cys64 at the N-terminus, in conjunction with His472, function as primary acceptors for electrons from NADPH *via* FAD, and that the electrons are then transferred to Cys497-Sec498 at the C-terminus for the reduction of oxidized thioredoxin in the mammalian thioredoxin reductase.

Key words: redox regulation, selenocysteine, site-directed mutagenesis, thioredoxin, thioredoxin reductase.

Thioredoxin reductase (TrxR), which exists as a homodimeric flavoenzyme, is a member of the pyridine nucleotide disulfide oxidoreductase family (*1*) and catalyzes the reduction of oxidized thioredoxin (Trx), with NADPH as the electron donor. Trx is a well-known electron donor to various systems, including proteins such as ribonucleotide reductase, transcription factors (*2*, *3*) and Trx-dependent peroxidases called peroxiredoxins (*4*, *5*). In addition, the Trx/TrxR system plays an important role in various biological functions such as regulation of apoptosis, immunomodulation, pregnancy and photosynthesis in diverse organisms (*6*). Although bacterial TrxRs have been well characterized and the crystal structure of *Escherichia coli* TrxR has been determined, mammalian TrxRs have been cloned relatively recently (*7*, *8*). Structural features predicted from the primary structure of the mammalian TrxR, which has a subunit size of 55 kDa, are totally different from those of the bacterial type, the size of which is 35 kDa. Although the mammalian TrxR has a marked similarity to glutathione reductase (*9*), it contains a rare amino acid, selenocysteine (Sec or U), at the penultimate position in the C-terminal extension, which has the sequence -Gly-Cys-Sec-Gly (*10*, *11*). Mammalian mitochondrial TrxR has also been cloned quite recently and was shown also to contain a Sec residue in the same C-terminal sequence (*12*, *13*). Since Sec is encoded by the UGA codon, which is generally recognized as a stop codon, a specific mechanism must operate to permit the incorporation of Sec at the position encoded by the UGA codon (*14*, *15*). All mRNAs encoding mammalian proteins that contain Sec have a specific element called a selenocysteine insertion sequence (SECIS) in the 3′-untranslated region (*14*, *15*). In a previous paper, we reported the

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[2] To whom all correspondence should be addressed Tel: +81-6-6879-3420, Fax: +81-6-6879-3429, E-mail: proftani@biochem.med.osaka-u.ac.jp

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); GR, glutathione reductase; IAA, iodoacetic acid sodium salt; Sec or U, selenocysteine; SECIS, selenocysteine insertion sequence; Trx, thioredoxin; TrxR, thioredoxin reductase.

functional expression of a rat TrxR in mammalian cells and showed that the SECIS element-mediated Sec incorporation is essential for TrxR activity (*16*).

Mammalian TrxRs have two reactive cysteine residues with the sequence -Cys-x-x-x-x-Cys- in the N-terminal FAD domain, which is conserved in glutathione reductase (*17*), lipoamide dehydrogenase (*18*), trypanotione reductase (*19*), and mercuric ion reductase (*20*). Site-directed mutagenesis studies of the cysteine residues clearly showed that each cysteine is essential for the acceptance of electrons from NADPH *via* FAD in the latter enzymes (*21–24*). Titration and pre-steady-state kinetics also indicate that the TrxR isolated from human placenta forms a thiolate-flavin charge transfer complex followed by reduction of the flavin, as has been demonstrated for lipoamide dehydrogenase or glutathione reductase (*9*).

The amino acid sequence of TrxR from the malaria parasite *Plasmodium falciparum* (pfTrxR) shows a high similarity to mammalian TrxR and has a subunit size of 59 kDa (*25*). This protein has the conserved N-terminal FAD domain, but contains two Cys residues in the C-terminal domain with the sequence -Cys-x-x-x-x-Cys-Gly, which differs from -Gly-Cys-Sec-Gly in the mammalian sequence. Mutants in which any one of the cysteines has been replaced by alanine lack Trx-reducing activity, but retain some 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB)-reducing activity (*26*). It has been shown that the C-terminal cysteines in pfTrxR play a role in redox communication with the active-site disulfide in the N-terminal domain (*27*, *28*).

Recently, the essential role of the Sec residue in the TrxR catalytic activity has been shown by replacing the Sec residue with Cys or Ser, and a redox communication between Cys-Sec at the C-terminus and the active-site disulfide at the N-terminus has been proposed in the mammalian TrxRs (*29–31*). However, the requirement of the Cys residue adjacent to the Sec residue in the TrxR activity and the mechanism of the electron transfer from NADPH to the substrate remain unclear. To clarify the catalytic mechanism of the mammalian TrxR, we introduced several mutations into the conserved N-terminal two cysteines and C-terminal histidine on the basis of the active-site structure of glutathione reductase (*32*) and into the Cys and Sec residues at the C-terminal extension in the rat TrxR cDNA. Some of these mutants were produced in baculovirus/insect cells and purified. Their kinetic properties and susceptibilities to the inhibitors were then compared with those of the authentic wild-type enzyme containing the Sec residue. Our findings show that both Cys and Sec residues at the C-terminal redox center are essential for the reduction of thioredoxin and that these residues function coordinately with the active site sulfhydryls at the N-terminus in the mammalian TrxRs.

EXPERIMENTAL PROCEDURES

Materials—Sodium [^{75}Se] selenite was obtained from Amersham. NADPH was obtained from Oriental Yeast (Tokyo). Recombinant *Escherichia coli* Trx was purchased from Promega. Bovine insulin was obtained from Bayer. Aurothioglucose, gold(III) chloride, glutathione (oxidized form) (GSSG), and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma. Sodium iodoacetate (IAA) and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Nakalai Tesque (Kyoto). Other reagents were of the highest grade available.

Cell Culture—COS-1 cells, a monkey kidney simian virus 40 (SV40) transformed cell line, were maintained in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin and 100 μg/ml streptomycin supplemented with 10% heat-inactivated fetal bovine serum (GIBCO/BRL) at 37°C in an atmosphere of 95% air and 5% CO_2. Culture of *Spodoptera frugiperda* (Sf21) cells and manipulations of the baculovirus were performed according to the procedures described by Piwnica-Worms (*33*). Sf21 cells were maintained in Grace's medium containing 3.3 mg/ml yeastolate, 3.3 mg/ml lactoalbumin hydrolysate, and 50 μg/ml gentamycin supplemented with 10% heat-inactivated fetal bovine serum (GIBCO/BRL) at 27°C.

Site-Directed Mutagenesis—The replacement of amino acid residues in the rat TrxR cDNA (*16*) by site-directed mutagenesis was performed according to the method of Kunkel (*34*) as described previously (*35*). The substitutions in the mutant TrxRs used in this work are summarized in Fig. 1. The putative active cysteines, Cys59 and Cys64, were changed to Ser individually, and the products were designated as C59S and C64S, respectively. The putative active-site base His472 was replaced by Ala (H472A). The C-terminal Cys497 was replaced by Ser (C497S). Sec498 was changed to either Cys (U498C) or Ser (U498S). In addition, Cys497-Sec498 was changed to Sec-Cys (CU498UC) or Ser-Ser (CU498SS). Those substitutions were confirmed by DNA sequencing using an automated sequencer (DSQ 1000, Shimadzu).

Expression of Wild-Type and Mutant TrxRs in COS-1 Cells—The wild-type TrxR cDNA and all mutant cDNAs were ligated into a pSVK3 expression vector (Pharmacia). This vector enabled the efficient expression of the cDNA in COS-1 cells, in which large T antigen is constitutively expressed to enhance the expression of the gene with the SV40 promoter/enhancer. These cDNAs were transiently transfected into COS-1 cells by electroporation (Gene Pulser, Bio-Rad).

Overproduction and Purification of Wild-Type and Mutant TrxRs—For the overproduction of TrxRs by the baculovirus/insect cell system, the wild-type cDNA and mutant cDNAs were ligated into the baculovirus transfer vector pVL1393 (Invitrogen) and cotransfected with Baculogold (Pharmingen) into Sf21 cells using Lipofectin (GIBCO/BRL). Purification of the TrxR proteins overproduced in Sf21 cells was carried out essentially as described previously (*16*). Excess FAD was added to the cell homogenate prior to the chromatographic steps. The contaminating TrxR-like activity from Sf21 cells was separated by use of a Blue Sepharose (Pharmacia) column as a passed-through fraction. An authentic TrxR was purified from a lysate of COS-1 cells which had been transfected on a large scale with the rat TrxR cDNA, designated as ΔTR2, which encodes the wild-type TrxR but lacks the 3′-untranslated region after the SECIS element. The transfectants with the ΔTR2 showed about 2-fold higher TrxR activity than those with the full-length wild-type TrxR cDNA as reported previously (*16*). The homogeneity of all proteins was assessed by SDS-PAGE.

Western Blotting—Samples (20 μg) were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane (Schleicher & Schuell) under semi-dry conditions by

using a Trans-blot (Bio-Rad). After blocking by incubation with 4% skim milk in Tris-buffered saline (20 mM Tris/HCl, pH 8.0/0.15 M NaCl) for 12 h at 4°C, the membrane was incubated with an antibody against human TrxR (*16*) at a 1:2,000 dilution for 2 h at room temperature, then washed with Tris-buffered saline containing 0.05% Tween 20. It was further incubated with peroxidase-conjugated goat anti–rabbit IgG (1:4,000, Organon Teknika) for 2 h, then washed, and peroxidase activity was detected by the chemiluminescence method using an ECL kit (Amersham).

Enzyme Activity—To determine TrxR activity, the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)- and Trx-reducing activities were used. The measurement of the Trx-reducing activity in the cell lysate was started by addition of 10 μl of enzyme source to 490 μl of buffer A (50 mM Tris/HCl, pH 7.5, 1 mM EDTA) containing 2 μM *E. coli* Trx, 500 μg/ml insulin, and 200 μM NADPH. The mixture was incubated at 37°C for 20 min, followed by termination by the addition of 500 μl of 6 M guanidine hydrochloride containing 1 mM DTNB. Trx-dependent insulin reduction by NADPH was determined by subtracting the amount of insulin reduction in the absence of Trx from that in the presence of Trx. To analyze the kinetic parameters for the Trx-reducing activity, purified TrxR proteins were added to assay mixtures containing varying concentrations of *E. coli* Trx, 200 μM NADPH, and 1 mM GSSG as a final electron acceptor in buffer A, and decrease in absorbance at 340 nm was monitored at 37°C (ε_{340} = 6.2 $mM^{-1}\cdot cm^{-1}$). To measure DTNB-reducing activity, samples were added to the assay mixtures containing varying concentrations of DTNB and 200 μM NADPH in buffer A, and the increase in absorbance at 412 nm was monitored at 25°C (ε_{412} = 13.6 $mM^{-1}\cdot cm^{-1}$). TrxR activity was defined as U/mg protein (1 U is equivalent to 1 μmol of NADPH oxidized per min) as described by Holmgren and Bjornstedt (*2*).

Transhydrogenase activity was measured at 25°C in buffer A in the presence of 20 μM NADPH by observing the initial rate of formation of thio-NADPH at 395 nm, as described previously (*36*), using an absorption coefficient of 1.13×10^4 $M^{-1}\cdot cm^{-1}$. One unit is defined as the appearance of 1 μmol of thio-NADPH per min.

Labeling of TrxR with ^{75}Se and Autoradiography—COS-1 cells transfected with mutant or wild-type TrxR cDNAs were cultured in medium containing sodium [^{75}Se]selenite (0.3 μCi/ml) for 3 days. The supernatants of the cell homogenates were subjected to SDS-PAGE, and the gel was then dried and subjected to autoradiography.

C-Terminal Sequence Analyses—The purified TrxR proteins were loaded on to Zitex membranes (*37*) and subjected to automated C-terminal degradation with a protein-sequencing system (Hewlett Packard, G1006A), according to the manufacturer's instructions. Although peaks derived from Ser and Cys from the intact protein emerged at the same positions on a chart, they could be distinguished by oxidizing Cys with performic acid before transferring the protein to the membrane. Thus, unambiguous data could be obtained by analyzing both intact and oxidized proteins.

Spectral Analyses—Spectral analyses of the oxidized forms of the purified TrxRs (2.2 mg/ml) were performed under aerobic conditions. Spectra of the reduced forms were obtained after 15 s of incubation with a 5-fold molar excess of NADPH to FAD. The spectra were recorded from 300 to 700 nm with a spectrophotometer (UV-3100PC, Shimadzu) at 20°C.

Inhibition Studies—For inhibition studies, effects of compounds on DTNB-reducing and transhydrogenase activities were measured. Aurothioglucose and gold(III) chloride dissolved in buffer A were prepared immediately before use. One minute after adding the compounds to the assay mixtures, which contained 400 ng/ml SecWT or 1 μg/ml U498C, the reaction was initiated by mixing with the substrate. Alkylating compounds, 1-chloro-2,4-dinitrobenzene (CDNB) dissolved in 100% ethanol and sodium iodoacetate (IAA) dissolved in buffer A were prepared prior to use. These compounds and the enzyme, 20 μg/ml SecWT or 250 μg/ml U498C, were incubated in buffer A in the presence of 200 μM NADPH for varying periods of time at room temperature, and the residual activity in the aliquots was assayed. The assay mixtures contained 1 mM DTNB and 200 μM NADPH for the DTNB-reducing activity and 50 μM thio-NADP and 20 μM NADPH for the transhydrogenase activity.

Protein Assay—Protein concentrations were determined using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

RESULTS

Enzyme Activities and the Incorporation of ^{75}Se in Mutant TrxRs Produced in COS-1 Cells—Since mammalian TrxR contains two catalytically important domains, one in the N-terminus and the other in the C-terminus, various mutations in rat TrxR cDNA were introduced by site-directed mutagenesis as summarized in Fig. 1. Mutations C59S, C64S, and H472A are in residues that are predicted to form the catalytic center based on structural similarity to glutathione reductase (*32*). The other mutations, U498C, U498S, C497S, CU498SS, and CU498UC, are in the C-terminal sequences, which are unique to the mammalian TrxR and are predicted to be essential for Trx-reducing activity. These mutant cDNAs as well as the wild-type cDNA were transiently transfected into COS-1 cells by electroporation (Fig. 2). The lysate from the transfected cells with the wild-type cDNA exhibited high TrxR activity, which was augmented by supplementation with sodium selenite as previously reported (*16*). However, none of the mutant cDNA-transfected cells showed any increment in TrxR activity, even on supplementation with sodium selen-

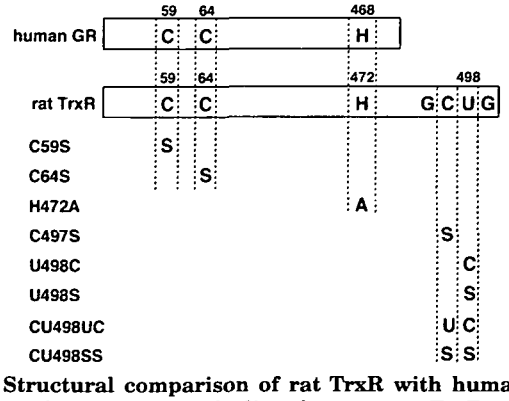

Fig. 1. **Structural comparison of rat TrxR with human glutathine reductase (GR) and a list of mutant rat TrxRs used in this study.**

ite. Thus, all mutants appeared not to have detectable TrxR activity in the cell homogenates (Fig. 2A). It is noteworthy that the TrxR activities of the C59S and C64S transfectants appeared to be lower than that of the control cells. Western blotting confirmed that the same levels of TrxR proteins were expressed in COS-1 cells for all transfectants (Fig. 2B). These transfected cells were incubated with sodium [^{75}Se] selenite for 3 days and radioactivity incorporated into the proteins was examined by autoradiography. Incorporation was detected at the position corresponding to the TrxR protein in the wild-type, C497S, CU498UC, C59S, C64S, and H472A, but not in U498C, U498S, or CU498SS (Fig. 2C). These data indicate that all the altered residues are required for Trx-reducing activity. Since CU498UC showed no increment in the TrxR activity despite the fact that it contains a Sec residue (Fig. 2, A and C), the position of Cys497 and Sec498 in the three-dimensional structure of TrxR might also be important for the activity.

C59S and C64S Would Function as Dominant Negative Mutants—The TrxR activities of C59S and C64S transfectants appeared to be much lower than that of the control COS-1 cells in Fig. 2A. To clarify this phenomenon, COS-1 cells were transiently co-transfected with wild-type TrxR cDNA and each of the mutant cDNAs of C59S, C64S, H472A, C498S, and U498S. Since transfection of ΔTR2, which encodes the wild-type rat TrxR cDNA but lacks the 3′-untranslated region downstream of the SECIS element (*16*), exhibits higher TrxR activity than the original wild-type cDNA and, hence, is beneficial to evaluate small activity changes in COS-1 cells, we used the ΔTR2 for the production of wild-type TrxR. Co-transfection with C59S or C64S and ΔTR2 suppressed TrxR activity in a dose-dependent manner compared with the transfection with ΔTR2 alone (Fig. 3A). In contrast, the suppression of activity was not observed in the transfectants expressing H472A, C497S, or U498S. Western blotting confirmed that in all cases, the levels of TrxR proteins in COS-1 cells expressing both the wild-type TrxR cDNA and the mutant cDNA were higher than those expressing the wild-type cDNA alone (Fig. 3B). These results indicate that inactive C59S and C64S acted in a dominant negative manner, presumably by forming heterodimers with the wild-type subunit intrinsic to the COS-1 cells.

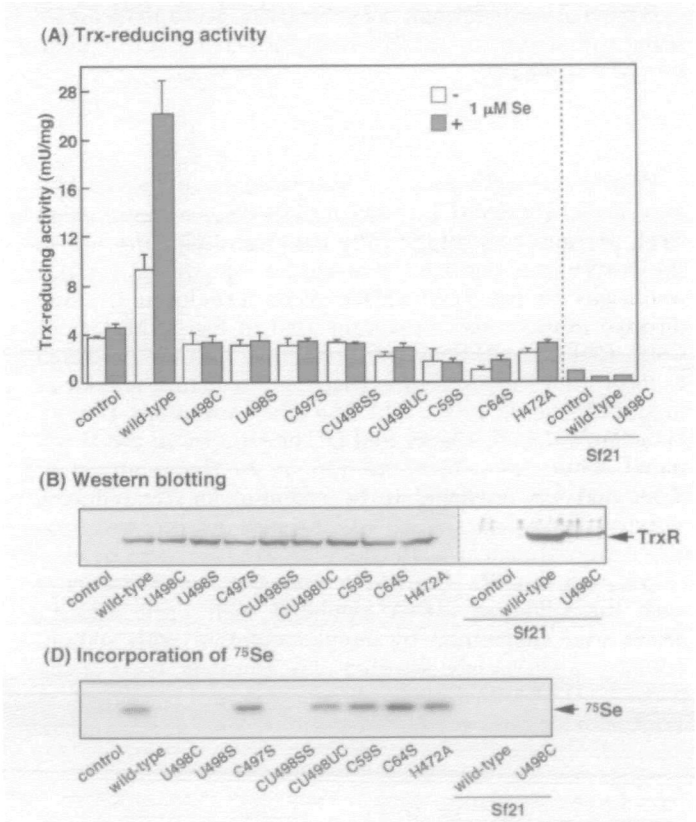

Fig. 2. **Characterization of wild-type and mutant TrxRs transiently expressed in the COS-1 cells and produced in the baculovirus/insect cells.** (A) Trx-reducing activity was measured in cell lysates prepared from COS-1 cells that had been transiently transfected with 50 μg of each cDNA indicated and from the Sf21 cells infected with baculovirus carrying the wild-type and U498C cDNAs. After transfection, the COS-1 cells were incubated for 3 days in the absence (open columns) or presence of 1 μM sodium selenite (solid columns) in culture medium. Data are presented as the means ± SD of triplicate experiments. (B) The cell lysates (20 μg) were subjected to Western blot analyses using the antibody against human TrxR. (C) Incorporation of ^{75}Se into the TrxR proteins in COS-1 cells and Sf21 cells expressed wild-type and mutant TrxRs. After transfection of the COS-1 cells and virus infection of the Sf21 cells, the cells were incubated in the medium containing sodium [^{75}Se] selenite (0.3 μCi/ml) for 3 days. The cell lysates (20 μg) were separated on SDS-PAGE and subjected to autoradiography.

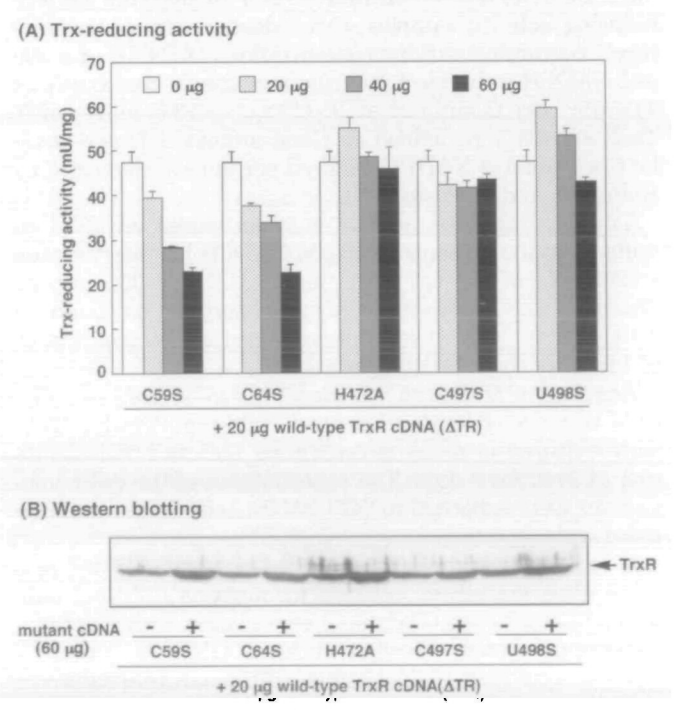

Fig. 3. **Co-transfection of the wild-type TrxR cDNA and each mutant cDNA in the COS-1 cells.** (A) The COS-1 cells were co-transfected with 20 μg TrxR cDNA designated as ΔTR2, which is a wild-type rat TrxR cDNA lacking the 3′-untranslated region after the SECIS element (*16*), and a varying dose of each mutant cDNA, C59S, C64S, H472A, C497S, or C498S. After transfection, the COS-1 cells were incubated for 3 days in the presence of 1 μM sodium selenite, and the Trx-reducing activities in cell lysates were measured. Data are presented as means ± SD of triplicate experiments. (B) The cell lysates (20 μg) expressing only wild-type cDNA or co-expressing wild-type cDNA and a mutant cDNA (60 μg) were subjected to Western blot analyses using the antibody against human TrxR.

TrxRs Produced in Baculovirus/Insect Cells Do Not Contain Sec—To purify TrxR proteins on a large scale, we overproduced the wild-type and U498C in the baculovirus/insect cell system. The cell lysates of virus-infected insect cells showed no increment in the TrxR activity in spite of the presence of large amounts of TrxR proteins (Fig. 2, A and B). Incorporation of ^{75}Se into the TrxR protein was not detected in cells that had been infected with the virus carrying the wild-type cDNA, even though the SECIS element was present in the 3′-untranslated region (Fig. 2C). We thus speculate that the protein produced in this system terminated at the UGA codon and no Sec residue was incorporated. This wild-type TrxR produced in the baculovirus/insect cells system was designated as WT.

We also produced and purified C59S, C64S, U498C, and U498S from the baculovirus/insect cell system. The purity of all proteins was confirmed by SDS-PAGE (data not shown). To identify the C-terminus of the WT and mutant enzymes which were overproduced in the insect cells, the C-terminal amino acids sequences were analyzed using the protein sequencer. The C-terminal amino acids in WT, C59S, and C64S were all Cys, and the penultimate amino acids were all Gly (data not shown) as expected, indicating that these proteins lack two amino acid residues, Sec and Gly, at their C-termini. These data indicate that these proteins were unable to incorporate Sec and terminated at the UGA codon in this system, even though the SECIS element was present in the cDNA. On the other hand, the C-terminal amino acids of U498C and U498S were found to be Gly, consistent with the C-terminal end of the predicted sequence, because the nonsense codons were converted to corresponding sense codons in these mutant cDNAs. For these reasons, an authentic TrxR which contained a Sec residue, designated as SecWT, was purified from COS-1 cells transfected with wild-type rat TrxR cDNA (*16*). These recombinant proteins were characterized as described below.

Spectral Analyses of the Enzymes—We next analyzed the absorption spectra of the purified WT and mutant enzymes (Fig. 4). The oxidized forms of WT, U498C, and U498S in the absence of NADPH exhibited a yellow-brown color with peaks at 460 and 380 nm, which are characteristic of oxidized flavin. When a 5-fold excess of NADPH was added, the color immediately changed to brick-red and a broad peak at 530 nm was observed, which corresponds to a flavin thiolate charge-transfer complex, as has been observed in human placental TrxR (*9*) and PfTrxR (*38*). C59S and C64S exhibited red shift and blue shift, respectively, and their whole absorption spectra were also altered compared with other enzymes. Since the isolated form of C59S showed a brick-red color, corresponding to the reduced form, the mutant remains in the flavin thiolate charge-

TABLE I. **Trx- and DTNB-reducing activities of SecWT, WT and mutant TrxRs.** Trx-reducing activity was assayed in buffer A containing 200 μM NADPH, 1 mM GSSG as a final electron acceptor, and 50 μM *E. coli* Trx, and the decrease in absorbance at 340 nm was monitored at 37°C. DTNB-reducing activity was assayed in buffer A containing 200 μM NADPH and 1 mM DTNB, and the increase in absorbance at 412 nm was monitored at 25°C. N.D.: not detected.

Enzyme	Trx-reducing activity (U/mg)	DTNB-reducing activity (U/mg)
SecWT	41	18
WT	N.D.	0.29
U498C	0.014	0.68
U498S	N.D.	0.50
C59S	N.D.	N.D.
C64S	N.D.	N.D.

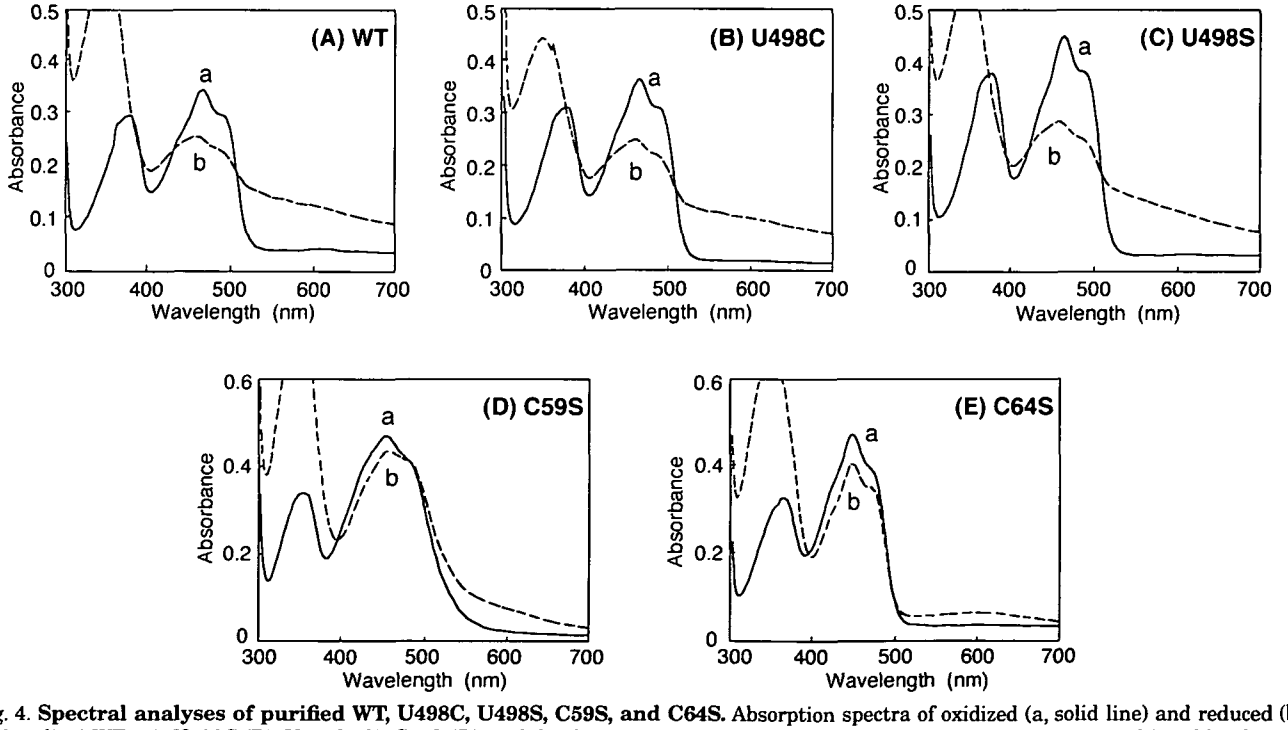

Fig. 4. **Spectral analyses of purified WT, U498C, U498S, C59S, and C64S.** Absorption spectra of oxidized (a, solid line) and reduced (b, broken line) WT (A), U498C (B), U498S (C), C59S (D), and C64S (E) were measured from 300 to 700 nm. Reduction was achieved by the addition of a 5-fold molar excess of NADPH to FAD to the enzyme solution.

transfer complex without NADPH. On the other hand, C64S, which maintained a bright yellow color, remained in the oxidized form, even in the presence of NADPH, indicating that the mutant was unable to form a charge-transfer complex. Although the same analysis was not carried out for SecWT due to the unavailability of sufficient protein for the assay, detailed spectral analyses of purified TrxR from human placenta provided essentially the same profiles as described in this study using recombinant enzymes (*9*).

Enzymatic Properties of WT and Mutant TrxRs in Comparison with SecWT—All the purified TrxR proteins overproduced in insect cells were assayed for reduction of Trx and DTNB and their activities were compared with SecWT. Table I shows values for enzymatic activity using either 50 μM *E. coli* Trx or 1 mM DTNB as a substrate. Of these proteins, only U498C showed Trx-reducing activity, although it was about three orders magnitude lower than that of SecWT. On the other hand, significant levels of DTNB-reducing activities, 2 to 4% of the SecWT, were observed for WT, U498C, and U498S. C59S and C64S were unable to catalyze the reduction of either Trx or DTNB. None of these enzymes exhibited GSSG-reducing activity (data not shown).

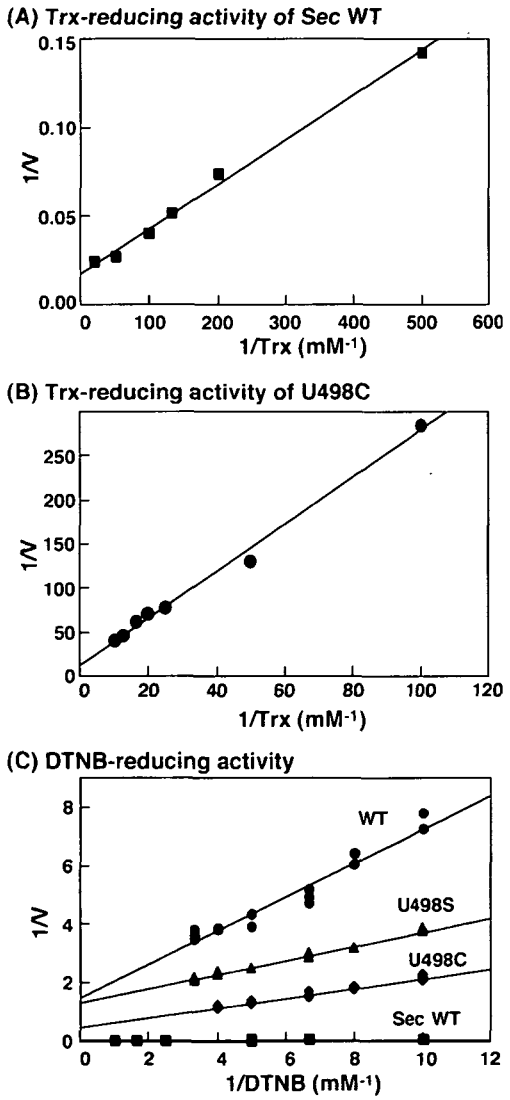

Fig. 5. **Kinetic analyses of Trx-reducing activity and DTNB-reducing activity of SecWT, WT, U498C, and U498C.** (A) and (B) Trx-reducing activity was assayed in a buffer A containing 200 μM NADPH, 1 mM GSSG as a final electron acceptor, and varying concentrations of *E. coli* Trx. The decrease in absorbance at 340 nm was monitored at 37°C. (C) DTNB-reducing activity was assayed in buffer A containing 200 μM NADPH and varying concentrations of DTNB. The increase in absorbance at 412 nm was monitored at 25°C. A double reciprocal plot was performed for the Trx-reducing activity of SecWT (A) and U498C (B) *versus* Trx concentrations and for the DTNB-reducing activity of WT, U498S, U498C, and SecWT *versus* DTNB concentrations (C).

TABLE II. **Kinetic parameters for Trx- and DTNB-reducing activities by SecWT, WT and mutant TrxRs.** Kinetic parameters were obtained from Fig. 5.

Enzyme	V_{max} (U/mg)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Trx-reducing activity				
SecWT	55	14	6,070	7.2×10^6
U498C	0.077	200	8.5	7.1×10^2
DTNB-reducing activity				
SecWT	19	100	2,100	3.4×10^5
WT	0.67	390	74	3.2×10^3
U498C	2.0	340	220	1.1×10^4
U498S	0.76	190	84	7.5×10^3

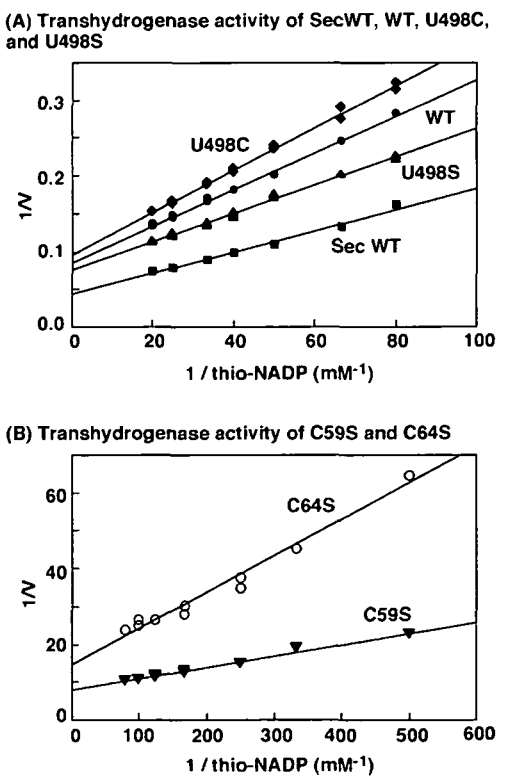

Fig. 6. **Kinetic analyses of transhydrogenase activity of SecWT, WT, U498C, U498C, C64S, and C59S.** Transhydrogenase activity was assayed in buffer A containing 20 μM NADPH and varying concentrations of thio-NADP. The increase in absorbance at 395 nm was monitored at 25°C. A double reciprocal plot was performed for the transhydrogenase activity of SecWT, WT, U498C, and U498C (A) and C64S and C59S (B) *versus* thio-NADP concentrations.

For kinetic analyses of these enzymes, double reciprocal plots for Trx- and DTNB-reducing activities were carried out (Fig. 5). The kinetic parameters for Trx- and DTNB-reducing activities of SecWT, WT, U498C, and U498S are summarized in Table II. The k_{cat} value of U498C for *E. coli* Trx was about 700-fold lower than that of SecWT, while the K_m value was about 15-fold higher. K_m values for DTNB were similar for all enzymes, but the k_{cat} value of U498C was one order lower and those of WT and U498S were about 30-fold lower than that of SecWT. This indicates that the Sec residue is essential for the reduction of Trx but not DTNB, although the Sec may enhance DTNB-reducing activity.

Transhydrogenase Activity of Wild-Type and Mutant Enzymes—Disulfide oxidoreductases are known to possess pyridine nucleotide transhydrogenase activity, which catalyzes the NADPH- or NADH-dependent reduction of thio-NADP$^+$ in the absence of a substrate (*39*). Since TrxR is a member of the disulfide oxidoreductase family, it also catalyzes the reaction of NADPH and thio-NADP$^+$ to NADP$^+$ and thio-NADPH. Double reciprocal plots and the calculated kinetic parameters for the transhydrogenase reaction catalyzed by WT and the mutants, as well as SecWT, are presented in Fig. 6 and Table III, respectively. The k_{cat} and the K_m values of WT, U498C, and U498S for thio-NADP$^+$ were comparable to those of SecWT. However, those of C59S and C64S were negligible compared with those of SecWT. These data signify that both Cys residues in the N-terminal FAD domain play a primary role in accepting a proton from NADPH *via* FAD and that the Sec residue in the C-terminus is not important for the reaction.

Inhibitory Effects of Gold Compounds on SecWT and U498C—Organic gold compounds such as aurothioglucose are widely used in the treatment of rheumatoid arthritis, whereas inorganic gold(III) chloride is not used for therapy because of its high toxicity. Since aurothioglucose and gold(III) chloride are potent inhibitors of selenoproteins (*40*), we compared the susceptibilities of SecWT and U498C to these compounds. Aurothioglucose at submicromolar concentations inhibited the DTNB-reducing activity of SecWT in the presence of NADPH. However, the DTNB-

TABLE III. **Kinetic parameters for transhydrogenase activity by SecWT, WT and mutant TrxRs.** Kinetic parameters were obtained from Fig. 6.

Enzyme	V_{max} (U/mg)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)
SecWT	23	32	2,500	1.3×10^6
WT	12	28	1,300	7.6×10^5
U498C	10	29	1,100	6.6×10^5
U498S	13	24	1,400	9.8×10^5
C59S	0.13	4	14	6.2×10^1
C64S	0.068	6	7.5	1.9×10^1

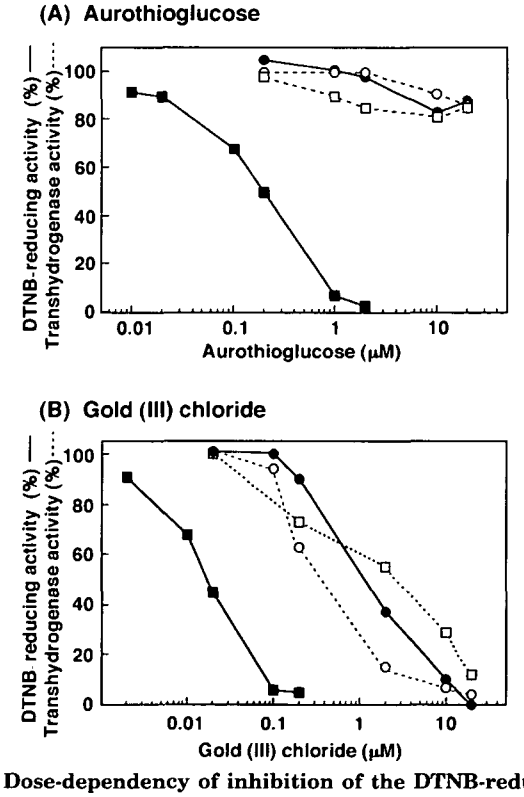

Fig. 7. **Dose-dependency of inhibition of the DTNB-reducing activity and transhydrogenase activity of SecWT and U498C by aurothioglucose and gold(III) chloride.** DTNB-reducing activity of Sec WT (closed squares) and U498C (closed circles) and transhydrogenase activity of Sec WT (open squares) and U498C (open circles) were assayed 1 min after adding varying concentrations of aurothioglucose (A) and gold (III) chloride (B). The activities with water instead of the gold compounds are defined as 100%.

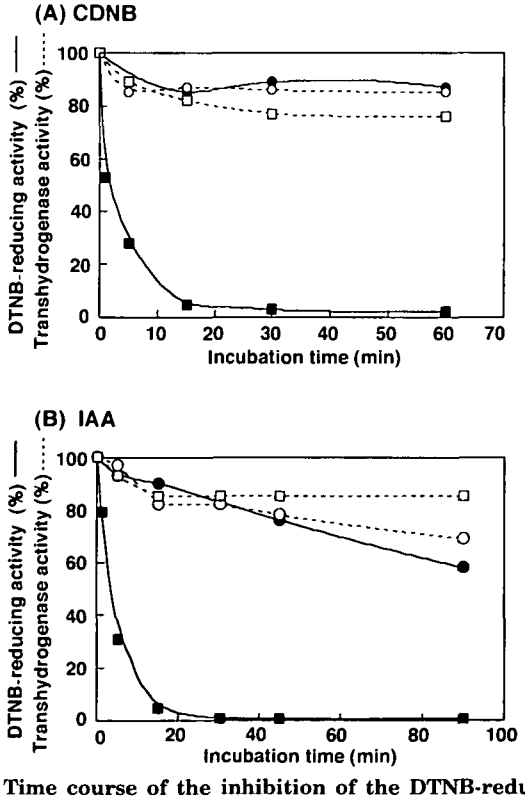

Fig. 8. **Time course of the inhibition of the DTNB-reducing activity and transhydrogenase activity of SecWT and U498C by CDNB and IAA.** The enzymes were incubated with CDNB (A) and IAA (B) in the presence of 200 μM NADPH for varying minutes at room temperature. DTNB-reducing activity of Sec WT (closed squares) and U498C (closed circles) and transhydrogenase activity of Sec WT (open squares) and U498C (open circles) were assayed using 10 μM CDNB or 100 μM IAA for Sec WT, and 1 mM CDNB or 2 mM IAA for U498C.

reducing activity of U498C, and transhydrogenase activities of both SecWT and U498C were barely affected by 20 μM aurothioglucose (Fig. 7A). Gold(III) chloride inhibited the DTNB-reducing activity of SecWT at 20 nM, but about two orders higher concentrations were required to inhibit U498C. The transhydrogenase activities of these enzymes were inhibited at the same extent by gold(III) chloride in the presence of NADPH (Fig. 7B). Inhibitory effects of the gold compounds were not observed in the absence of NADPH during preincubation (data not shown). Gold(III) chloride is known to inhibit not only selenoproteins but also glutathione reductase (*40*). We conclude from these data that this compound also modifies the N-terminal sulfydryl groups in the mammalian TrxR and glutathione reductase. Our data suggest that the Sec residue is a main target of the inhibitory action of these compounds, and that the sulfhydryl groups in the N-terminal active site are much less reactive to them.

Effects of Alkylating Agents on SecWT and U498C—CDNB irreversibly inhibits mammalian TrxR in the presence of NADPH and induces NADPH oxidase activity by alkylating both Cys and Sec in the C-terminus (*41*, *42*). We investigated the effects of two alkylating agents, CDNB and IAA, on the SecWT and U498C enzyme activities (Fig. 8). DTNB-reducing activities of SecWT were rapidly inhibited by 10 μM CDNB or 100 μM IAA in the presence of NADPH, whereas those of U498C remained unaffected even by millimolar concentrations of these compounds. The transhydrogenase activities of both enzymes were hardly inhibited by these compounds. These results suggest that the Sec residue is markedly sensitive to alkylating agents, and the sulfhydryls in the N-terminal domain are resistant to them under these conditions.

DISCUSSION

In this study, we introduced several mutations into residues of TrxR that were presumed from its structural similarity to glutathione reductase (*32*) to form the catalytic center and might be essential for the Trx-reducing activity in the sequences, which are unique to the mammalian TrxR (Fig. 1). We found that all residues mutated here, Cys59, Cys64, His472, Cys497, and Sec498, are essential for the TrxR activity and the order of Cys-Sec in the sequence is also important for the catalytic mechanism, since all mu-tants, including CU498UC, showed no increment in TrxR activities in the COS-1 cells (Fig. 2A). These data clearly demonstrate that these amino acid residues involved in the catalysis of mammalian TrxR.

To investigate the precise reaction mechanism of mammalian TrxR, we overproduced wild-type and several mutant enzymes in a baculovirus/insect cell system and purified them. Infection of the virus carrying wild-type cDNA, which contains a SECIS element in the 3′-untranslated region, produced a protein with neither Trx-reducing activity nor incorporation of Sec (Fig. 2). Analyses of the C-termini demonstrated that WT, C59S, and C64S ended with cysteine, indicating that the proteins terminated at the UGA codon. Since the UGA encoding Sec generally functions as a stop codon, a special machinery would be required for the incorporation of Sec into polypeptides (*14*). It is conceivable that the synthesis of Sec-tRNA^{Sec} or the machinery to incorporate Sec into mammalian polypeptides does not function or is insufficient in the baculovirus/insect cell system.

Of the purified mutant enzymes examined, only U498C exhibited a slight but significant Trx-reducing activity, suggesting that the Sec residue was essential for the efficient reduction of Trx and that Cys, but not Ser, partially compensated the role of Sec. On the other hand, some DTNB-reducing activities were observed for WT, U498C, and U498S, although two amino acid residues, Sec-Gly, at the C-terminus are missing in WT. These results suggest that the Sec residue is not essential for the reduction of DTNB. However, the Sec residue has a role in the enhancement of the DTNB-reducing activity, because the k_{cat} values of these enzymes were significantly lower than that of SecWT (Table II). A mutant human TrxR (*43*) and a mutant rat TrxR (*29*), which corresponds to our U498C, were produced in the baculovirus/insect cell system and the *E. coli* system, respectively. Their Trx-reducing activities were about two orders of magnitude lower than that of Sec-containing enzyme. While the mutant human TrxR (*43*) and U498C in this work showed higher K_m values for Trx than the Sec-containing enzyme, the mutant expressed in *E. coli* showed a 10-fold lower K_m for Trx compared to the Sec-containing enzyme (*29*). This discrepancy might be due to the difference in the system used for expression of the mutant enzymes.

While WT, U498C and U498S retained comparable transhydrogenase activity to SecWT, C59S and C64S exhibited neither substrate-reducing activity nor transhydrogenase activity. These data suggest that the Cys residues in the N-terminal redox center, but not the residues in the C-terminal redox center, are essential for the acceptance of electrons from NADPH *via* FAD. Spectral analyses of these mutants show that C59S remains in the reduced form when isolated, and C64S remains in the oxidized form, even in the presence of NADPH (Fig. 4), which suggest that Cys64 is the primary proton acceptor from NADPH *via* FAD, and that Cys59 is required for the transhydrogenase reaction in the final step. Figure 9 presents a schematic model, which is similar to one proposed recently (*31*), for the electron transfer between the N-terminal and the C-terminal redox centers, which was constructed based upon our results and those in the literature. While the spectral properties of C59S and C64S remained constant for a long time after the addition of NADPH, the spectra of WT, U498C, and U498S were converted to the reduced forms immediately after NADPH was added, and returned to the oxidized forms slowly under aerobic conditions (data not shown). Collectively, these observations and the change in absorption spectrum of the native TrxR isolated from human placenta (*9*) suggest that the C-terminal redox center (Cys-Sec) is not in close contact with the isoalloxazine moiety of the flavin bound to the protein.

The TrxR activities of C59S and C64S transfectants were lower than that of the control cells (Fig. 2A). The transfection of C59S or C64S cDNA together with wild-type TrxR cDNA showed lower TrxR activity than that of wild-type TrxR cDNA alone (Fig. 3). These results suggest that the newly produced polypeptide from C59S or C64S cDNA formed a heterodimer with that of endogenous TrxR in the COS-1 cells or with the active TrxR produced from the transfected wild-type TrxR cDNA. These phenomena and the structural assumption suggest that the Cys497-Sec498

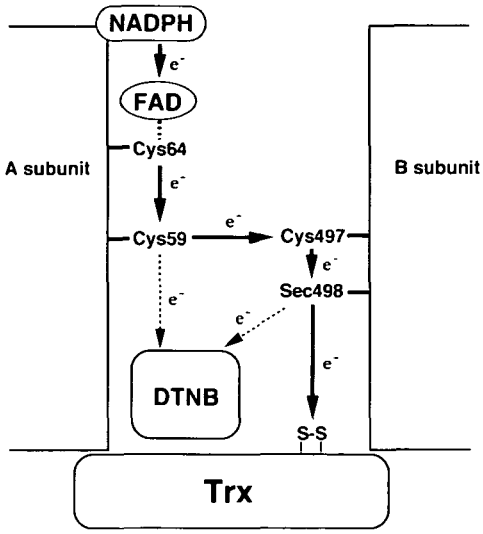

Fig. 9. **Postulated brief model of electron transfer in the mammalian TrxR.** Arrows indicate the route of electron flow from NADPH to oxidized Trx. Dotted arrows indicate the route of electron flow in the final step of the DTNB-reducing reaction.

in the C-terminal redox center of one subunit interacts with Cys59 and Cys64 in the N-terminal redox center of the other subunit during the Trx-reducing reaction. These data also suggest that, in the heterodimer with wild-type TrxR subunit and C59S or C64S subunit, electrons are unable to be transferred from the N-terminal active site in the wild-type subunit to the intact C-terminal redox center in the mutant subunit, which results in the dominant negative feature of C59S and C64S. Since heterodimers formed with H472A, C497S or U498S did not show a dominant negative effect (Fig. 3), only mutations in the N-terminal redox reactive cysteines appears to influence the intact C-terminal redox center. Further structural analysis is required to clarify this point.

Since the gold compounds and alkylating agents affected the Sec-containing wild-type enzyme more severely than U498C (Figs. 7 and 8), the Sec residue appears to be a primary accessible site for oxidized Trx. Holmgren *et al.* (*31*) proposed that selenolate anion attacks the disulfide of Trx, since the selenolate anion is much more nucleophilic than the thiolate anion. Recent reports have shown that the C-terminal consecutive Cys and Sec residues form a selenenylsulfide (S-Se) and the S-Se can be converted to fully reduced state, thiol and selenol, by NADPH from N-terminal active cysteines, Cys59 and Cys64, in the mammalian TrxRs (*30*, *31*). From these and our data, we postulate the model of electron transfer in the mammalian TrxR shown in Fig. 9. In this model, Cys 64 accepts electrons from NADPH *via* FAD and transfers them to Cys59 in the A subunit. The electrons are then transferred to Cys497 and finally to Sec498 in the B subunit. For the Trx-reducing reaction, oxidized Trx accepts the electrons only from Sec498. The remaining DTNB-reducing activity in the mutants at the C-terminal redox center (Tables I and II and Fig. 4) indicates that DTNB can access the N-terminal redox center directly and accept electrons from Cys59. Thus a steric effect of the N-terminal active center may prevent access of Trx. Crystallographic analysis of the enzyme is required to conclude the actual role of the Sec in the C-terminal redox center.

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