Mammalian thioredoxin reductases (TrxR) are dimers homologous to glutathione reductase with a selenocysteine (SeCys) residue in the conserved C-terminal sequence -Gly-Cys-SeCys-Gly. We removed the selenocysteine insertion sequence in the rat gene, and we changed the SeCys⁴⁹⁸ encoded by TGA to Cys or Ser by mutagenesis. The truncated protein having the C-terminal SeCys-Gly dipeptide deleted, expected in selenium deficiency, was also engineered. All three mutant enzymes were overexpressed in Escherichia coli and purified to homogeneity with 1 mol of FAD per monomeric subunit. Anaerobic titrations with NADPH rapidly generated to homogeneity with 1 mol of FAD per monomeric mammalian TrxR. However, only the SeCys⁴⁹⁸ late-flavin charge transfer complex characteristic of mammalian TrxRs are homologous to glutathione reductase and have a C-terminal elongation containing a conserved selenocysteine residue in the penultimate position (Fig. 1). The SeCys residue has been implicated in the enzyme mechanism since more than 4 electrons per subunit are required to reduce completely the FAD of the oxidized enzyme (16). Furthermore, the SeCys residue is alkylated with loss of activity only after reduction by NADPH (12, 17, 18), and free SeCys is released by carboxypeptidase digestion with loss of catalytic activity only from the NADPH-reduced enzyme (12). The SeCys residue is also the target of the irreversible inhibitor 1-chloro-2-nitrobenzene (19) as shown by peptide analyses (17).

The cDNAs for human (20) and rat (12) thioredoxin reductase contain an in-frame TGA codon corresponding to the penultimate SeCys residue in the protein and a conserved stem-loop structure folded as an SECIS motif about 200 base pairs downstream in the 3′-untranslated region (Fig. 1). The SECIS element is required for decoding the mRNA UGA codon, which otherwise confers termination, to incorporate selenocysteine by a species-specific mechanism (21–23). Attempts to express a putative human placenta thioredoxin reductase in E. coli (11) before it was discovered that the TGA encodes SeCys (20) rather than translation termination resulted in a protein with an identical subunit size as the native enzyme (55 kDa) on SDS-PAGE but inactive since it lacked FAD (11). Truncation by UGA acting as a stop codon, therefore, suggested that the C-terminal SeCys-Gly dipeptide may have a function in protein folding or FAD binding (24).

In this paper we have used site-directed mutagenesis of the rat enzyme to examine the role of the selenocysteine residue. We have replaced the SeCys by either the chemically similar Cys or the redox-inactive Ser, and we also engineered the truncated protein DesSeCys⁴⁹⁸-Gly⁴⁹⁹ resulting from the UGA acting as a stop codon. We have purified all three mutant proteins to homogeneity in high yield from E. coli. Physicochemical and enzymatic analysis demonstrated that only the SeCys⁴⁹⁸ → Cys enzyme showed activity in reduction of thioredoxin with a major loss of Km. Native rat TrxR reduced hydrogen peroxide with a high Km value for H2O2 but this activity was absent in the Cys mutant. However, addition of thioredoxin...
doxin and free selenocystine strongly stimulated activity lowering the $K_m$ value for H$_2$O$_2$ dramatically. Our results demonstrate that selenium is essential for the catalytic activities of TrxR and directly involved in the mechanism of the enzyme. A preliminary report of this work has been published (25).

**EXPERIMENTAL PROCEDURES**

**Materials—**NADPH, DTNB, hydrogen peroxide, PMSF, lipoic acid, sodium selenite, bromochloroindolyl phosphate, nitro blue tetrazolium, alkaline phosphatase-labeled anti-rabbit IgG, and Triton X-100 were from Sigma. Selenocystine was from Serva (Heidelberg, Germany). Recombinant human thioredoxin was purified as described (26). Human placenta, calf liver, and rat liver thioredoxin reductase were purified to homogeneity by previously described methods (3, 4, 27). DEAE-Sepharose, PhastGel for electrophoresis, and 2',5'-ADP-Sepharose were from Amersham Pharmacia Biotech. Restriction enzymes were from Promega. LipofectAMINE reagent and tissue culture media were from Life Technologies, Inc.

**Expression of Recombinant Rat Thioredoxin Reductase in COS Cells**—The desired region of 1.8 kilobase pairs (Fig. 1b) starting at nt 113 of the rat TrxR cDNA previously cloned and sequenced (12) was amplified by PCR using 5'-TCG AAA GCT AGC AAT GAA TGA-3' as the forward primer and 5'-AAC ATG ATA CAC ATT ACA TAG CTT GAA GGC-3' as the reverse primer. The translation start methionine codon is shown in boldface. The primers contained NheI or BamHI restriction site, respectively, to facilitate subcloning of the amplified fragment into a pcDNA3.1 (+zeo - ) vector at the corresponding sites. The construct was transferred into COS-7 cells by using LipofectAMINE™ reagent for transient expression. In 75-cm$^2$ plastic flasks, 2,5 × 10$^6$ cells were plated and incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (complete medium) at 37 °C. When cells were ~70% confluent they were incubated with the transfection mixture composed of 5 μg of DNA, 12 μl of LipofectAMINE™ reagent (2 mg/ml), and serum-free medium. After 24 h incubation with the transfection mixture containing 5 μl LipofectAMINE reagent (2 mg/ml), and serum-free medium.

**Assays of Enzyme Activity**—Enzyme activity of thioredoxin reductase were examined with a Shimadzu UV160U spectrophotometer using previously developed methods (3, 4). Reduction of 5 mM DTNB was measured in 100 mM potassium phosphate buffer, 1 mM EDTA, pH 7.5, 1 mM PMSF, and 0.1% Triton X-100 and sonicated. Following centrifugation, the supernatant fraction was loaded on a column of DEAE-Sepharose (2.5 × 15 cm). TrxR was eluted with a linear gradient from 0 to 0.5 M NaCl in 50 mM potassium phosphate, pH 7.5 and 1 mM EDTA (1000 ml each). Fractions with DTNB reducing activity were pooled and dialyzed against 50 mM potassium phosphate, pH 7.5, 1 mM EDTA and applied to a column of 2',5'-ADP-Sepharose (1.5 × 7 cm) equilibrated with this buffer. The bound enzyme was eluted with a linear gradient of 0.05-0.3 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA (400 ml each). Fractions containing pure mutant TrxR were dialyzed against 50 mM potassium phosphate, pH 7.5, 1 mM EDTA and stored frozen at −80 °C.

**Western Blotting**—Proteins were separated on SDS-polyacrylamide PhastGel with a gradient 8–25 and transferred to a nitrocellulose membrane and probed with rabbit antiserum raised against rat TrxR (1: 8000 dilution). For immunoblot detection, the membranes were incubated with alkaline phosphatase-labeled anti-rabbit IgG and developed with bromochloroindolyl phosphate/nitro blue tetrazolium substrates.

**Construction of TrxR Mutant Proteins**—The open reading frame of the rat TrxR cDNA from nt 123 to 1649 was amplified by PCR. The sense primer was 5'-TCA ACC ATG GAT GAC TCT AAA GAT GCC CCT-3' (methionine start codon in bold type). The antisense mutant primer 1 was 5'-CAA CAG QAT CCA CAC TGG GCC TTA ACC QCA GCA GC-3'; the antisense mutant primer 2 was 5'-CAA CAG QAT CCA CAC TGG GCC TTA ACC QCA GC-3', and the antisense mutant primer 3 was 5'-CAA CAG QAT CCA CAC TGG GCC TTA ACC QTA GCA GC-3'. The mutated sites are underlined. The corresponding Se-codon (TGA) was thus altered to Cys (TGC), Ser (TCA), or stop (TAA) codon, respectively (Fig. 1c). The amplified PCR products were subcloned into pGEM-T vector and sequenced to confirm the expected mutagenesis by an ALF sequencer (Amersham Pharmacia Biotech) as described (12). Note that resequencing the original cDNA corrected the sequence by 3 additional nt, so that position 1646 in the old sequence now corresponds to 1649 (see below).

**Overexpression of TrxR Mutant Proteins in E. coli**—The cDNA insert was excised from the pGEM-T vector at the introduced NcoI and BamHI site and ligated into a PET-3d vector. The resulting plasmids were used to transform E. coli BL21(DE3)pLysS cells. Cells were grown in LB medium containing carbencillin (100 μg/ml) and chloramphenicol (34 μg/ml) at 37 °C and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at ~0.6 A$_{600}$nm for 4 h. The cells were harvested by centrifugation and washed with 50 mM phosphate buffer. Thioredoxin (5 μM) was added and incubated at 37 °C for 30 min at 3000 rpm at 4 °C in a Sorvall RC5 centrifuge, the supernatant fraction was loaded on a column of DEAE-Sepharose (2.5 × 15 cm). TrxR was eluted with a linear gradient from 0 to 0.5 mM NaCl in 50 mM potassium phosphate, pH 7.5 and 1 mM EDTA (1000 ml each). Fractions with DTNB reducing activity were pooled and dialyzed against 50 mM potassium phosphate, pH 7.5, 1 mM EDTA and applied to a column of 2',5'-ADP-Sepharose. The bound enzyme was eluted with a linear gradient of 0.05-0.3 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 400 ml each). Fractions containing pure mutant TrxR were dialyzed against 50 mM potassium phosphate, pH 7.5, 1 mM EDTA and stored frozen at −80 °C.

**Purification of Recombinant TrxR Mutant Proteins**—E. coli cells were resuspended in 5 volumes of cold 50 mM potassium phosphate, 2 mM EDTA, pH 7.5, 1 mM PMSF, and 0.1% Triton X-100 and sonicated.

**Selenocysteine Is Essential for Mammalian Thioredoxin Reductase**

Fig. 1. a, schematic protein structure of rat thioredoxin reductase based on its homology to glutathione reductase (12). b, native rat TrxR cDNA with TGA codon for selenocysteine and selenocysteine insertion sequence (SECIS) element in the 3′-untranslated region (3′-UTR). c, engineered cDNA for expression of selenium-deficient TrxR mutants in E. coli. Structures are not drawn to scale.

Cys codon (TGA) was thus altered to Cys (TGC), Ser (TCA), or stop (TAA) codon, respectively (Fig. 1c). The amplified PCR products were subcloned into pGEM-T vector and sequenced to confirm the expected mutagenesis by an ALF sequencer (Amersham Pharmacia Biotech) as described (12). Note that resequencing the original cDNA corrected the sequence by 3 additional nt, so that position 1646 in the old sequence now corresponds to 1649 (see below). Overexpression of TrxR Mutant Proteins in E. coli—The cDNA insert was excised from the pGEM-T vector at the introduced NcoI and BamHI site and ligated into a PET-3d vector. The resulting plasmids were used to transform E. coli BL21(DE3)pLysS cells. Cells were grown in LB medium containing carbencillin (100 μg/ml) and chloramphenicol (34 μg/ml) at 37 °C and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at ~0.6 A$_{600}$nm for 4 h. The cells were harvested by centrifugation and washed with 50 mM phosphate buffer. Thioredoxin (5 μM) was added and incubated at 37 °C for 30 min at 3000 rpm at 4 °C in a Sorvall RC5 centrifuge, the supernatant fraction was loaded on a column of DEAE-Sepharose (2.5 × 15 cm). TrxR was eluted with a linear gradient from 0 to 0.5 mM NaCl in 50 mM potassium phosphate, pH 7.5 and 1 mM EDTA (1000 ml each). Fractions with DTNB reducing activity were pooled and dialyzed against 50 mM potassium phosphate, pH 7.5, 1 mM EDTA and applied to a column of 2',5'-ADP-Sepharose (1.5 × 7 cm) equilibrated with this buffer. The bound enzyme was eluted with a linear gradient of 0.05-0.3 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA (400 ml each). Fractions containing pure mutant TrxR were dialyzed against 50 mM potassium phosphate, pH 7.5, 1 mM EDTA and stored frozen at −80 °C.

Western Blotting—Proteins were separated on SDS-polyacrylamide PhastGel with a gradient 8–25 and transferred to a nitrocellulose membrane and probed with rabbit antiserum raised against rat TrxR (1: 8000 dilution). For immunoblot detection, the membranes were incubated with alkaline phosphatase-labeled anti-rabbit IgG and developed with bromochloroindolyl phosphate/nitro blue tetrazolium substrates.

**Assays of Enzyme Activity**—Enzyme activities of thioredoxin reductase were examined with a Shimadzu UV160U spectrophotometer using previously developed methods (3, 4). Reduction of 5 mM DTNB was measured in 100 mM potassium phosphate buffer, 1 mM EDTA, pH 7.0, 0.1 mM NADPH, and 0.1 mg/ml bovine serum albumin. The reaction was followed by the increase of absorbance at 412 nm. Thioredoxin (5 μM) reduction was followed by coupling to insulin (160 μM) disulfide reduction (3, 27). For other substrates, the reaction mixtures contained 50 mM potassium phosphate, 2 mM EDTA, pH 7.0, and 0.2 mM NADPH.
For $\text{H}_2\text{O}_2$ reduction only the initial velocity during the 1st min was used for calculation of data. Reactions were initiated by adding enzyme to the sample cuvette and the same amount of buffer to the blank cuvette. Reaction rates were followed by the decrease in absorbance at 340 nm, resulting from oxidation of NADPH. The activity was calculated using a molar extinction coefficient of 6,200 $M^{-1} \cdot cm^{-1}$ for NADPH at 340 nm. Apparent $K_m$ values were calculated from Lineweaver-Burk plots of $1/v$ against $1/[S]$.

**Protein Analyses—**Thioredoxin reductase concentration was determined either by measuring the absorbance of flavin at 460 nm using a molar extinction coefficient of 10,000 $M^{-1} \cdot cm^{-1}$ or using the absorbances at 280 nm and subtracting the absorbance at 310 nm, using a molar extinction coefficient of 100,900 $M^{-1} \cdot cm^{-1}$. Protein sequence analysis employed generation of tryptic peptides which were purified by high pressure liquid chromatography on a RPC C2/C18 SC 2.1/10 column and sequenced on a PROCIe protein sequencer (12). Absorbance spectra were recorded on a Shimadzu UV 160U UV-visible spectrophotometer.

**Anaerobic Titration with NADPH—**Solutions of thioredoxin reductase were placed in cuvettes covered with a rubber septum. The NADPH solution was placed in a separate glass vial covered with a rubber septum. The content was bubbled with argon, purified through another needle air was evacuated. This anaerobic treatment lasted for 30 min. To the TrxR samples aliquots from the NADPH solution were added via a gas-tight Hamilton syringe. Spectra were recorded after each addition using the spectrophotometer at 25 °C.

**RESULTS**

**Expression of Active Rat Thioredoxin Reductase in COS-7 Cells—**We previously cloned and sequenced a 2.2-kilobase rat TrxR cDNA containing the open reading frame and a 3′-untranslated region of 560 nucleotides containing a SECIS element (12). To show that our rat TrxR cDNA was functional, it was cloned in the mammalian expression vector pcDNA 3.1/zeo(−). In transiently transfected COS-7 cells, the total TrxR activity was increased 70% compared with the control cells transfected with the empty vector. By Western blotting, extracts of the cells transfected with rat TrxR expression plasmid showed a new positive band (Fig. 2), which corresponded to TrxR at 55 kDa, plus another positive band with higher molecular weight of unknown origin. This experiment proved that the rat cDNA directed synthesis of full-length and active enzyme. However, only trace amounts of rat enzyme in a background of COS-7 cell TrxR was obtained from this expression system, which precludes analysis of the effects of mutation by enzyme kinetics and other methods requiring pure proteins in milligram quantities.

**Expression and Purification of Mutant Thioredoxin Reductase in E. coli—**When the 2,156-nucleotide fragment from the rat TrxR cDNA was cloned in an expression plasmid and trans-
milligram quantities of the pure mutant proteins allows us to
examine their catalytic properties with different substrates for
mammalian thioredoxin reductase. As shown in Table II, only
the SeCys498
3
Cys TrxR showed activity with Trx-S 2 in re-
ducing insulin disulfides, whereas the other mutant proteins
were inactive. DTNB which is used as a substrate to assay the
enzyme activity (3, 4) showed 4.7% activity with the SeCys498
3
Cys enzyme compared with calf liver TrxR and with a
similar
Km
value (data not shown). The Cys mutant enzyme
showed 9.1, 2.7, and 2.1% activity of wild type TrxR in selenite,
lipoic acid, or selenocystine reduction reactions, respectively.
The other two mutants had very low activity with all these
substrates. Despite the presence of an N-terminal redox-active
disulfide in TrxR identical to that of glutathione reductase (Fig.
4), neither the native enzyme nor any of the mutant proteins
reduced GSSG (data not shown).

![Table II](http://www.jbc.org/)

### Table II. Activity of purified native and mutant mammalian thioredoxin reductase enzyme with different substrates

| Substrate       | Wild Type TrxR | SeCys498 Cys TrxR | Cys Mutant Enzyme |
|-----------------|----------------|------------------|-------------------|
| DTNB            | 100%           | 4.7%             | 9.1%              |
| Lipoic acid     | 5.5%           |                  | 2.7%              |
| Selenocystine   | 4.5%           |                  | 2.1%              |
| Selenite        | 3.2%           |                  | 0.8%              |

**Fig. 4.** Results of amino acid sequence analysis and alignment of rat SeCys498 → Cys TrxR and human erythrocyte glutathione reductase (GR) based on primary and secondary structure. 1st line is secondary structure of the rat TrxR predicted using the program "nnpredict"; 2nd line is the rat TrxR sequence deduced from cDNA sequence; 3rd line is human glutathione reductase sequence, and 4th line is secondary structure of human glutathione reductase taken from Ref. 13. The nomenclature is used as follows: (e) β-sheet and (H) α-helix. The three functional regions FAD-binding motif, redox-active disulfide, and NADP(H)-binding motif are boxed. The amino acid sequences determined by Edman degradation are underlined. The C-terminal extension of the rat TrxR includes a substituted Cys 498 for SeCys. This C-terminal cysteine pair is accessible and is not linked by disulfide in the native enzyme since both the cysteine residues were alkylated by 4-vinylpyridine and released as separate phenylthiohydantoin-cysteine from nonreduced C-terminal peptide, as shown in the lower panel.
Comparison of the Rat Liver and the Rat SeCys 498 Cys Enzyme in Reduction of Human Thioredoxin—Since the mutant enzyme with the SeCys to Cys replacement was active, we made a detailed comparison with the wild type enzyme prepared from rat liver. As shown in Fig. 7 the apparent \( K_m \) and \( k_{cat} \) values for the wild type enzyme at pH 7.5 were 3.3 mM and 2,500 \( \text{min}^{-1} \) or close to previously reported values (3). In contrast the apparent \( K_m \) of the SeCys498 3 Cys mutant was lower than that of the wild type or 0.4 mM, whereas the \( k_{cat} \) was 14.3 \( \text{min}^{-1} \) or 0.6% of the selenocysteine containing wild type enzyme.

The activities in thioredoxin-dependent reduction of insulin catalyzed by the wild type and SeCys498 \( \rightarrow \) Cys TrxR were determined over the pH range 4.2 to 10.5. The SeCys498 \( \rightarrow \) Cys enzyme exhibited a broad pH optimum of 7 (Fig. 8). The sharp drop in activity of the mutant enzyme at high pH may reflect enzyme denaturation. The overall profile was similar to that of wild type TrxR which, however, exhibited a pH optimum of 7 (Fig. 8). This shift in activity to the alkaline side should be related to the replacement of the selenocysteine with a cysteine in a putative active site. At a physiological pH of 7.0, the selenol (nominal \( pK_a \) of 5.3) should be fully ionized, and the replacement by a thiol (nominal \( pK_a \) of 8.25) should be partially protonated, suggesting that the ionic state of the SeCys 498 is directly involved in the rate-determining step of catalysis. The results provide a strong argument of the involvement of the selenocysteine residue in the mechanism of electron transfer.

Activity with Hydrogen Peroxide—We examined the activity of rat and human placenta thioredoxin reductase with \( \text{H}_2\text{O}_2 \), and the enzymes directly reduced this peroxide (Fig. 9). To measure activity, only the initial rate during the 1st min was used. Assuming Michaelis-Menten kinetic rather than a second order reaction, the apparent \( K_m \) value for \( \text{H}_2\text{O}_2 \) was 2.5 mM and the \( k_{cat} \) was 100 \( \text{min}^{-1} \). In contrast, none of the three mutant proteins showed any peroxide reductase activity (\( \leq 1% \) of the wild type enzyme). As shown in Fig. 9 the SeCys498 3 Cys mutant enzyme activity with \( \text{H}_2\text{O}_2 \) was not stimulated by addition of thioredoxin. However, addition of 4.5 mM selenocystine together with 5 mM Trx strongly stimulated the \( \text{H}_2\text{O}_2 \) reducing activity. The activity increased to around 20% that of the natural enzyme. Also, most important, the apparent \( K_m \) value for \( \text{H}_2\text{O}_2 \) was reduced from 2.5 to 0.1 mM. This effect of free selenocyst(e)ine acting to catalyze the reaction via an unknown intermediate was previously seen with the human enzyme alone (6, 10). In this case the SeCys 498 \( \rightarrow \) Cys enzyme the effect was evident only with thioredoxin present.
DISCUSSION

Recent studies have demonstrated multiple isoenzymes of thioredoxin reductase in mammalian tissues including three forms in the cytosol (28). In addition mitochondrial thioredoxin reductase (29–31) is present as three isoforms probably by differential splicing (32). All these isoforms share the conserved C-terminal sequence -Gly-Cys-SeCys-Gly and the basic glutathione reductase-like structure outlined in Fig. 4, whereas they are different in the N-terminal regions.

In this study we have examined the result of replacing the penultimate selenocysteine residue in rat thioredoxin reductase (12) by cysteine or serine or removing it together with the C-terminal glycine residue. Our results show that folded FAD containing mutant enzymes in high yield were obtained by expression in E. coli. Titration with NADPH under anaerobic conditions demonstrated the appearance of a lower absorbance in the 460-nm region and a new long wave band at 540 nm in all three mutant enzymes. This is consistent with formation of a thiolate-flavin charge transfer complex originally observable but not interpreted in rat liver thioredoxin reductase (3) and extensively studied in the human placenta enzyme (16). The thiolate-flavin charge transfer arises from the flavin and the N-terminal redox-active disulfide which is fully conserved between glutathione reductase and thioredoxin reductase (11–13) (Fig. 4). The result is consistent with an intact first half-reaction for all mutant proteins involving transfer of electrons from NADPH to the N-terminal disulfide. The results also rule out a role for the C-terminal SeCys-Gly dipeptide in FAD binding or interaction with this coenzyme.

In contrast to the similar behavior of all three mutant enzymes in NADPH titrations, only the SeCys 498 → Cys mutant protein showed activity in reduction of thioredoxin. The results of the kinetic analysis showed a major 100-fold decrease in $k_{cat}$ with a shift of the pH optimum of 2 pH units. This is a strong argument in favor of the SeCys residue being directly involved in the active site mechanism of reduction of the active site disulfide (Cys-Gly-Pro-Cys) in thioredoxin (33). Electrons to reduce the oxidized SeCys as a selenenylsulfide will come from the reduced N-terminal disulfide in the other subunit of the

| Substrate                  | Enzyme activity (mol of NADPH oxidized/min/mole enzyme) |
|----------------------------|--------------------------------------------------------|
| Human Trx 5 μM             | 1,667 (100)                                            |
| DTNB 5 mM                  | 1,333 (100)                                            |
| Lipoic acid 1 mM           | 297 (100)                                              |
| Selenocysteine 45 μM       | 471 (100)                                              |
| Selenocysteine 45 μM       | 102 (100)                                              |
| Human Trx 5 μM             | 1.667 (100)$^a$                                        |
| DTNB 5 mM                  | 1.333 (100)                                            |
| Lipoic acid 1 mM           | 0.8 (2.7)                                               |
| Selenocysteine 45 μM       | 43 (9.1)                                                |
| Selenocysteine 45 μM       | 2.1 (2.1)                                               |

$^a$ Values in parentheses are percentage of calf liver thioredoxin reductase (CL-TrxR) activity.

$^b$ Taken from Ref. 4.

Fig. 7. Dependence of human Trx concentration for the activity of native rat thioredoxin reductase (top) and SeCys498 → Cys mutant thioredoxin reductase (bottom). Experiments were performed at pH 7.5 and 25 °C. Results are presented as Lineweaver-Burk plots.

Fig. 8. Activities of wild type and SeCys498 → Cys mutant thioredoxin reductases as a function of pH. Activity was assayed by Trx-dependent reduction of insulin. The reaction mixture contained 100 mM potassium phosphate, 5 mM EDTA, 0.1 mM insulin, 0.2 mM NADPH, and 5 μM human Trx (C63S/C72S) in a volume of 120 μl. The reaction was started by addition of the enzyme. After incubation 10 min at 37 °C, the reaction was broken by 500 μl of 8 M guanidine hydrochloride, 1 mM DTNB in 50 mM Hepes buffer, pH 7.6. The activity was calculated from the net absorbance at 412 nm as the formation of SH groups in insulin. The 100 mM potassium phosphate was adjusted to the indicated pH values with NaOH. Activities are expressed here as percentage of the highest activity.

Fig. 9. Activities of rat thioredoxin reductase mutants and comparison with wild type enzyme
Selenocysteine Is Essential for Mammalian Thioredoxin Reductase

Zhong, L., Arné, E. S. J., and Holmgren, A. (2000) Proc. Natl. Acad. Sci. U. S. A., in press.

A Human placenta TrxR

B SeCys498Cys TrxR

Fig. 9. Selenium requirement for mammalian thioredoxin reductase to reduce H$_2$O$_2$. A, human placenta thioredoxin reductase (59 nM) directly catalyzed reduction of H$_2$O$_2$. The inset shows a Lineweaver-Burk plot to determine apparent $K_m$ and $k_{cat}$ B, SeCys$_{498}$ → Cys mutant thioredoxin reductase (1,200 nM) did not reduce H$_2$O$_2$ even with human Trx present unless selenocysteine was added.

In a dimer arrangement, a head to tail structure as in glutathione reductase$^1$ (1) (Fig. 4). The decrease in $k_{cat}$ of substituting SeCys by Cys by 2 orders of magnitude is similar to results for E. coli formate dehydrogenase H where this substitution also resulted in more than 2 orders of magnitude reduction in catalytic activity (34). Also for rat type 1 iodothyronine deiodinase, a similar effect on catalytic activity by a SeCys to Cys substitution was reported (35). This lack of activity in the Ser mutant or DesSeCys$_{498}$-Gly$_{499}$ enzyme is consistent with a catalytic role of the SeCys residue as an electron acceptor and donor. Recently results from a human thioredoxin reductase Cys mutant expressed in baculovirus was published (36). Although the $K_m$ value for thioredoxin was reported to be higher for a Cys mutant than the wild type placenta enzyme and was reported as $\mu$M rather than $\mu$M probably due to a printing error, the large effect on $k_{cat}$ we have observed was also seen in this system. The $k_{cat}$ value for the wild type enzyme was low, however (36). We have recently demonstrated that both the SeCys residue and adjacent Cys residue are alkylated by the irreversible inhibitor 1-chloro-2-nitrobenzene (17). Furthermore, we have unpublished data demonstrating a selenenylsulfide linking these two residues in the wild type-oxidized enzyme.$^3$ This second C-terminal redox center is proposed to be formed in oxidation of the enzyme by thioredoxin. An additional role of the selenocysteine residue would then be structural since bonds between two adjacent Cys or SeCys residues are rare and not favored. The larger radius of the selenium atom in the SeCys residue may then be important. It is of particular significance in this study that both the Cys$_{497}$ and SeCys$_{498}$ residues were alkylated by vinylpyridine and detected as free phenylthiolyldiazain-derivatives in the Edman degradation (Fig. 4). This is consistent with the presence of two thiol groups left unoxidized to a disulfide in the SeCys$_{498}$ → Cys enzyme after purification.$^3$

All three mutant proteins were obtained in high yield in E. coli. We have recently crystalized the active rat SeCys$_{498}$ → Cys thioredoxin reductase and obtained crystals that diffract to better than 3 Å resolution,$^4$ and the structure of the mutant enzyme should soon be available. Preparations of the native selenocysteine-containing wild type enzyme yield enzyme with varying specific activities (4, 18, 36) probably as a result of varying degrees of loss of selenium (28, 36, 37). Recently, use of E. coli and gene fusion with engineered bacterial-type SECIS elements and coexpression with selA, selB, and selC genes have resulted in production of large quantities of selenocysteine-containing wild type enzyme with about 25% of the specific activity of the pure enzyme (38). The main protein in this preparation that remains to be separated (38) is the inactive truncated DesSeCys$_{498}$-Gly$_{499}$ enzyme we have studied.

The content of a catalytically active selenocysteine residue in mammalian thioredoxin reductase explains the previously surprising observation of the direct reduction of lipid hydroperoxides (10). In this paper we have also demonstrated that H$_2$O$_2$ is a substrate for human placenta thioredoxin reductase with a $k_{cat}$ of 100 $\times$ min$^{-1}$, which is about 3% that for the natural substrate thioredoxin (4). However, the $K_m$ for H$_2$O$_2$ was relatively high or 2.5 mM, which may mean that the enzyme under normal conditions is of relatively minor importance for removal of H$_2$O$_2$ compared with enzymes such as glutathione peroxidases or thioredoxin peroxidases (peroxiredoxins) (39). The hydroperoxidase activity may serve to protect the enzyme from self-inactivation by hydroxyl radical ions formed by oxygen univalent reduction.$^5$ An hypothesis involving the SeCys residue for regulation of activity has also recently been proposed (28). Our results clearly show that the selenocysteine is required for the activity since the SeCys$_{498}$Ser mutant as well as the DesSeCys$_{498}$-Gly$_{499}$ enzymes were inactive.

The Cys mutant enzyme showed undetectable activity with H$_2$O$_2$ by itself. However, addition of selenocysteine, the diselenide amino acid, and thioredoxin resulted in considerable activity in H$_2$O$_2$ reduction (Fig. 9). The apparent $K_m$ value for H$_2$O$_2$ was also much lower and potentially in a physiological range as a local concentration. The same stimulatory effect of selenocysteine in place of cysteine (23) this reaction is probably of no physiological significance. In fact with selenocyst(e)ine present, it is known that thioredoxin reductase and thioredoxin will give extensive redox cycling with oxygen (7) as is also the case for selenite (6) or selenodiglutathione (8) at (pM) concentrations. Thus, thioredoxin reductase with its selenocysteine residue is a major reason for the inability of cells to handle free selenocysteine; the other is obviously random incorporation of selenocysteine in place of cysteine (23).

The essential selenocysteine residue in thioredoxin reductase is almost certainly the target of therapeutic gold com-

$^3$ Zhong, L., Arné, E. S. J., and Holmgren, A. (2000) Proc. Natl. Acad. Sci. U. S. A., in press.

$^4$ L. Zhong, K. Persson, T. Sandalova, G. Schneider, and A. Holmgren, submitted for publication.

$^5$ T. Kerimov, S. Kuprin, and A. Holmgren, unpublished results.
Selenocysteine Is Essential for Mammalian Thioredoxin Reductase

pounds, such as gold thioglucose or auronofin which are powerful inhibitors of enzyme in vitro (40, 41) and in vivo (42). Also a number of chemotherapeutic drugs are also targeted to the enzyme (43). The absolute requirement of selenocyteine in the function of the mammalian thioredoxin system strongly suggests a mechanism for the essential role of this trace element in cell growth. It is well known that selenite is required for the growth of cells in tissue culture (44, 45). Furthermore, selenium has anticarcinogenic effects (46–48). The selenoprotein thioredoxin reductase is a cornerstone of cellular antioxidant defense and regulation of cell growth and differentiation through effects via thioredoxin. It is involved in synthesis of deoxyribonucleotides for DNA synthesis via the role of reduced thioredoxin as an electron donor for essential enzyme ribonucleotide reductase (2, 33). Selenite reduction to selenide is catalyzed by thioredoxin reductase (6), and the truncated protein expected in selenium deficiency is inactive in reduction of thioredoxin; it is yet unknown if this has any other biological role or is present in selenium-starved cells.

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Essential Role of Selenium in the Catalytic Activities of Mammalian Thioredoxin Reductase Revealed by Characterization of Recombinant Enzymes with Selenocysteine Mutations
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