Rat and Calf Thioredoxin Reductase Are Homologous to Glutathione Reductase with a Carboxyl-terminal Elongation Containing a Conserved Catalytically Active Penultimate Selenocysteine Residue*

(Received for publication, September 12, 1997, and in revised form, November 16, 1997)

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We have determined the sequence of 23 peptides from bovine thioredoxin reductase covering 364 amino acid residues. The result was used to identify a rat cDNA clone (2.19 kilobase pairs), which contained an open reading frame of 1496 base pairs encoding a protein with 498 residues. The bovine and rat thioredoxin reductase sequences revealed a close homology to glutathione reductase including the conserved active site sequence (Cys-Val-Asn-Val-Gly-Cys). This also confirmed the identity of a previously published putative human thioredoxin reductase cDNA clone. Moreover, one peptide of the bovine enzyme contained a selenocysteine residue in the motif Gly-Cys-SeCys-Gly (where SeCys represents selenocysteine). This motif was conserved at the carboxyl terminus of the rat and human enzymes, provided that TGA in the sequence GCC-TGG-TGA-GGT-TAA, being identical in both cDNA clones, is translated as selenocysteine and that TAA confers termination of translation. The 3′-untranslated region of both cDNA clones contained a selenocysteine insertion sequence that may form potential stem loop structures typical of eukaryotic selenocysteine insertion sequence elements required for the decoding of UGA as selenocysteine. Carboxypeptidase Y treatment of bovine thioredoxin reductase after reduction by NADPH released selenocysteine from the enzyme with a concomitant loss of enzyme activity.

Thioredoxin reductase from Escherichia coli has been extensively characterized (1), and a high resolution x-ray structure shows surprisingly large differences from the other members of the pyridine nucleotide-disulfide oxidoreductase family (8, 9). Thus, the subunits of about 35 kDa are smaller than the ~50-kDa subunits present in glutathione reductase from all species. Furthermore, the active site cysteine residues of E. coli TrxR are located in the NADPH domain and separated by two amino acids (Cys-Ala-Thr-Cys), in comparison with the active site in glutathione reductase, which is Cys-Val-Asn-Val-Gly-Cys and located in the FAD domain, suggesting convergent evolution (9). The structural features of TrxR from E. coli with a high specificity for its homologous Trx are also typical for TrxR from prokaryotes, lower eukaryotes like yeast, or plants (1, 8–10).

It has long been known that TrxR from mammalian cells has very different properties compared with that from E. coli and lower organisms (2–4). The enzymes from calf liver and thymus and rat liver were first purified to homogeneity and showed subunits with an M₉ of 58,000 (11, 12). The mammalian thioredoxin reductases including that of human placenta (13) are not only larger than the E. coli enzyme but have a very different and wide substrate specificity. Thus, the mammalian enzymes will reduce thioredoxin from different species (11), several low molecular weight disulfide substrates including DTNB used in assays (11, 12), or lipoic acid (14) as well as other nondisulfide substrates including selenodiglutathione (15), selenite (16), alloxan (17), or (most surprisingly) lipid hydroperoxides (18). The wide substrate specificity indicates an unusual structure of the active site, which is also demonstrated by the inhibition of mammalian TrxR by several drugs in clinical use including antitumor quinones (19, 20), nitrosoareas (21), or...
13-cis-retinoic acid (22). Furthermore, 1-chloro-2,4-dinitrobenzene selectively inactivated the reduced form of mammalian TrxR (23).

Recently, two proteins from transformed human cells having thioredoxin reductase activity, a 55-kDa subunit enzyme from a Jurkat T-cell line (24) and a 57-kDa subunit protein from a lung adenocarcinoma cell line (25), were shown to contain selenocysteine. A peptide sequence from the protein purified from the human T cell line (24) agreed with a putative human placental TrxR cDNA sequence showing homology to glutathione reductase but not prokaryotic TrxR (26). The putative human placental cDNA clone did not give any active enzyme, since expression in E. coli resulted in a protein of the correct Mr, which, however, did not incorporate FAD (26).

We have sequenced peptides from calf thymus and liver TrxR. Together with the sequence of the translated open reading frame of a rat TrxR cDNA clone, the close structural homology to glutathione reductase was evident, and the earlier published putative human cDNA clone could be confirmed. We found that a carboxyl-terminal motif containing cysteine and selenocysteine was conserved among human, rat, and bovine TrxR, and we present evidence that the carboxyl terminus is essential for the catalytic activity of the enzyme. Preliminary results have been reported (27).

**EXPERIMENTAL PROCEDURES**

**Materials**

2',5'-ADP-Sepharose and Q Sepharose were from Amersham Pharmacia Biotech. dl-Selenocystine was from Serva (Heidelberg). Trypsin came from Promega, and endoproteinase Lys-C was from Wako. Carboxypeptidase Y was kindly provided by Prof. Viktor Mutt (Karolinska Institutet, Stockholm). Recombinant human Trx was prepared as described (28). Chemicals for peptide sequencing were delivered by Perkin-Elmer, and chemicals for DNA sequencing were delivered by Amersham Pharmacia Biotech. All additional chemicals were of analytical grade or better, and sources have been given in previous publications (11, 12, 17).

**Purification of Thioredoxin Reductase**

Calf liver and thymus were obtained from a local slaughter house and stored frozen at −20 °C. TrxR was purified by the method described earlier (11, 12) with some modifications summarized as follows. Crude extract was acidified to pH 5.0, the neutralized supernatant was fractionated on a 20-ml Aminohexyl-Sepharose column, which was eluted with a 0–0.6 M NaCl gradient in TE buffer. This eluent and was then concentrated by centrifugation in a Microsep concentrator (Filtron, 10-kDa cut-off). Peptides were generated upon 10-fold dilution in 100 mM NH₄HCO₃, pH 8.0, and incubation at 37 °C overnight with either trypsin or lysine protease at a ratio of 1:10 (protease:TrxR), in the presence of 1 m guanidine hydrochloride. Peptides were separated using the Amersham Pharmacia Biotech SMART system with a C₂/C₁₈ Sephasil column and a linear gradient of 0 to 80% acetonitrile in water containing 0.1% trifluoroacetic acid during 90 min at a flow rate of 50 μl/min. Eluted peptides were detected at 215, 280, and 254 nm and fractionated by automatic peak detection using the absorbance at 215 nm. Peptides were then analyzed by peptide sequencing on a Procise Protein Sequencer (Applied Biosystems) according to the manufacturer's instructions.

**Analysis of Peptides from TrxR Labeled with Iodoacetic Acid Followed by 4-Vinylpyridine**

TrxR (150 μg) was dissolved in 80 mM Tris-HCl, 0.5 mM guanidine hydrochloride, 0.2 mM EDTA, pH 8.0, for 30 min at room temperature. The SH groups were carboxymethylated using 300 nmol of iodoacetic acid overnight in the dark at room temperature. The carboxymethylated enzyme was desalted by gel filtration (Fast desalting PC 3.2/10 column). This protein solution was added 720 nmol of NADPH. After an additional 15 min of incubation, the nascent SH groups were derivatized with 250 nmol of 4-vinylpyrindine for 4 h at room temperature in the dark. The mixture was desalted again, and 5 μl of endoproteinase Lys-C (2 μg/μl) was added. The digestion was carried out at 37 °C overnight. After the addition of another 5 μl of endoproteinase Lys-C and an additional incubation for 2 h, the resulting peptides were separated on a 1 ml RPC (C₂/C₁₈; SC2.1/10) column from Amersham Pharmacia Biotech. The column was eluted with a linear gradient from 0 to 80% acetonitrile in 1.0% trifluoroacetic acid over 190 min at a flow rate of 0.1 ml/min. The Edman procedure was used for analyzing the peptides on a Procise Protein Sequencer (Applied Biosystems).

**Carboxypeptidase Y Treatment of TrxR**

Carboxypeptidase Y (1 mg/ml) was dissolved in 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and stored for no more than 24 h at −20 °C prior to use to avoid autodigestion. EDTA was included to inhibit possible contaminant activities of yeast protease A or aminopeptidase.

**Assay of TrxR Activity**—TrxR activity was determined as the NADPH-dependent reduction of 5 mM DTNB or 5 μM human Trx and insulin dispensed by previously developed methods (12). One unit of enzyme activity is defined as the oxidation of 1 μmol of NADPH min⁻¹ at 25 °C (12).

**Inactivation with Carboxypeptidase Y**—To determine the time course of TrxR inactivation by carboxypeptidase Y, 2.7 μg of calf liver TrxR was preincubated with 8 nmol of NADPH in 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, at room temperature for 30 min in a total volume of 10 μl. To the reduced enzyme, 1 μl of carboxypeptidase Y (1 mg/ml) was added, and the sample was incubated at 37 °C. Aliquots (2.5 μl) were removed at various times and assayed for catalytic activity. Control incubations were identical except that carboxypeptidase and/or NADPH was not included.

**Analysis of Released Amino Acids**—Calf liver TrxR (105 μg) was diluted in 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, with 320 nmol of NADPH in a final volume of 380 μl. After preincubation for 30 min at
room temperature, 20 μl of carboxypeptidase Y (1 mg/ml) was added, and the mixture was incubated at 37 °C. After 30 min, 2 h, 4 h, and 24 h, 60-μl aliquots were withdrawn and stored at −20 °C. To monitor the effect of digestion, 0.5-μl aliquots from each sample were taken for analysis of TrxR activity, as described above. This showed that no more than 60% inactivation had occurred up to 24 h of incubation, and therefore an additional 10-μl carboxypeptidase Y was added to each 60-μl aliquot, and incubation was continued at 37 °C, for each of the samples for the same time period as before the second addition of carboxypeptidase Y, i.e. 30 min, 2 h, 4 h, and 24 h. These incubations were again terminated by moving the samples to −20 °C. For analysis of amino acids released after carboxypeptidase Y digestion, protein was removed from the low molecular weight fraction. To do this and to stop further digestion, samples were thawed and centrifuged in Millipore Ultrafree-MC filters (10-kDa cut-off). The filtrates containing buffer, salts, and released amino acid residues were lyophilized, 7 μl of 0.5 M DTT was added, and then the samples were subjected to amino acid analysis with ion exchange chromatography and ninhydrin derivatization using an Amersham Pharmacia Biotech 4151 Alpha Plus analyzer. Standard amino acids were analyzed under the same conditions.

Cloning and Sequencing of Rat TrxR cDNA

Using part of a bovine TrxR peptide with a unique amino acid sequence (KVMVLDFVTPTPLGTRCG), a rat cDNA clone was identified in GenBank™ searches as coding for rat thioredoxin reductase,² originally derived from rat neuroblastoma cells treated with nerve growth factor (31), and only partially sequenced. The clone was obtained from the Institute for Genomic Research (31), and to determine insert size and end sequences of the insert, M13 universal and M13 reverse oligonucleotide primers were used for polymerase chain reaction amplification of the insert as well as initial sequence reactions. Sequence determination of the complete insert was carried out using an automated laser fluorescent DNA sequencer (A.L.F., Amersham Pharmacia Biotech) according to protocols from the manufacturer, with subsequent primers based on the new sequence data.

RESULTS

Selenium Content of Mammalian Thioredoxin Reductase—We started structural studies of bovine (11) and rat (12) thioredoxin reductase several years ago with the aim of cloning the enzyme and to explain its unique structural characteristics (27).

Due to the recent report of selenium in TrxR isolated from transformed human cells (24), we analyzed TrxR, classically purified from calf thymus (11) to determine if this also contained selenium. We found that TrxR from calf thymus as well as human placenta contained 0.6 selenium atoms/58-kDa subunit, which was less than the corresponding value of 0.74 selenium atoms (24) or, as recently reported for TrxR from human placenta, 0.93 selenium atoms (32) per subunit. Since we found that human and calf TrxR contained the same amount of selenium, we continued our ongoing study of the calf enzyme, with a special emphasis on the structure of the enzyme and the significance of the selenium content for its catalytic properties.

Bovine TrxR Peptide Sequences—Sequence analysis of reduced and carboxymethylated TrxR from calf liver revealed a blocked NH₂ terminus. Interestingly, following incubation in 30% acetic acid, an NH₂-terminal sequence KDLPEPYDYDLIGGGSGGYD could be determined, which we later found starts four residues after the initiating methionine based on alignments to human and rat TrxR (see below).

Initial sequence information of TrxR purified from calf thy-

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² N. H. Lee, K. G. Weinstock, E. F. Kirkness, J. A. Earle-Hughes, R. A. Fuldner, S. Marmaras, A. Glodek, J. D. Gocayne, M. O. Adams, A. R. Kerlavage, C. M. Fraser, and J. C. Venter, GenBank™ accession number H34190.
mus was obtained by cleavage of the enzyme with trypsin or endoproteinase Lys-C followed by reverse phase HPLC and sequence determination of the resulting peptides. In total, we determined the sequence of 23 peptides, covering in total 364 reliable amino acid residues. None of these peptide sequences showed high homology with earlier determined thioredoxin thioredoxin reductase sequences from lower organisms. However, the close homology with glutathione reductase was evident for several of the peptides, especially to a probable glutathione reductase from C. elegans that is probably a thioredoxin reductase. The peptides also showed very high homology to the deduced amino acid sequence of a putative cDNA clone of human placental TrxR (26), thereby confirming this putative sequence, since all peptides were derived from purified native and enzymatically active thioredoxin reductase.

In a separate experiment, we selectively derivatized residues of calf liver TrxR that were reduced by NADPH. This was done by carboxymethylation of sulphydryl groups in the oxidized enzyme with unlabeled iodoacetic acid prior to incubation with NADPH and with subsequent alkylation of NADPH-dependently reduced residues with 4-vinylpyridine. This modified enzyme was then digested with endoproteinase Lys-C, and peptides were separated by reverse phase HPLC. Using this approach, several peptides labeled with 4-vinylpyridine could be detected by increased absorption at 254 nm (Fig. 1). One of these peptides (Fig. 1, peak A) had the determined sequence VMVLDFVTPTPLGTRCGLGGTCVNVGCIP. The COOH-terminal part of this peptide is identical to the active site of glutathione reductase, Cys-Val-Asn-Val-Gly-Cys (1). Both Cys residues had been derivatized with 4-vinylpyridine, indicating that only after incubation with NADPH were the nascent sulphydryl groups susceptible to alkylation. In the oxidized enzyme, a disulfide would be present. The unique NH₂-terminal part of this peptide, not homologous to glutathione reductase or other enzymes of this family, was subsequently used to identify a rat TrxR cDNA clone (see below).

A second peptide selectively labeled with 4-vinylpyridine upon incubation with NADPH (Fig. 1, peak C) had the sequence RSGNILQTGCGX, where X represents a pyridylethylated uncommon amino acid, eluting immediately after the standard Gly residue in the carboxyl-terminal region. An additional TrxR to reduce Trx seemed more susceptible to carboxypeptidase Y treatment than the capacity to reduce DTNB (Fig. 2A). The oxidized TrxR was resistant to attack of carboxypeptidase Y, solely incubation with NADPH did not inactivate the enzyme (Fig. 2A). The ability of TrxR to reduce Trx seemed more susceptible to carboxypeptidase Y treatment than the capacity to reduce DTNB (Fig. 2B), the latter compound used as a substrate in assays of the enzyme (3, 11).

Data obtained after amino acid analysis of residues released from calf liver TrxR upon carboxypeptidase Y treatment are presented in Fig. 3. After 30 min of digestion, carboxypeptidase Y had released free Gly and SeCys. The release of Gly continued to increase, consistent with the presence of more than one Gly residue in the carboxyl-terminal region. An additional amino acid residue could be detected that increased with time but did not co-elute with any of the amino acids known to be present at the carboxyl terminus of the enzyme (Fig. 3, peak X).

### TABLE I

| Peptide amino acid sequences | Comment |
|----------------------------|---------|
| NH₂-terminal peptide       |         |
| KDLFEPVDYDLHIIGGGGSGGYD     |         |
| Internal peptides          |         |
| LMHRQAALLLQQALDDRNYGGNEETVK|         |
| MTEAVQQNIALN               |         |
| VALR                       |         |
| KVTVENAYGEFVPHRIK          |         |
| IYSAEQFLIATGERPRLYGIPDK    |         |
| EYQQISSDFLQFLXY            |         |
| LLGFQIQDMAN                |         |
| IGEHQXEHGK                |         |
| FIRQFVIPK                 |         |
| VEQIEATGTPGRLVIA           |         |
| STDSDQTIEGEYNTVLLAIGRDAATRK|         |
| LELTPVIAQAGRLLAQ           |         |
| LYGSTVVK                  |         |
| CDYENVPTVFTPLEYGSCGSEEEK   |         |
| AVEK                       |         |
| FGEENVYHYSYFPWEWTIP       |         |
| CYAK                       |         |
| VVCNIK                     |         |
| CGLTK                      |         |
| DQDLSTIGHPVCAE             |         |
| Peptides selectively pyridylated after incubation with NADPH (see Fig. 1) | Containing glutathione reductase-like active site motif |
| VMVLDFVTPTPLGTRCGLGGTCVNVGCIP|         |
| RSGNILQTGCGX               |         |

* X denotes unclear residue at sequence determination. Some peptides were also longer than shown but were uncertain due to low yield with indistinct determination; hence, only reliable residues are shown.

* Sorted in order from the NH₂ terminus, as later revealed by the alignment shown in Fig. 6.

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3 J. Sulston, Z. Du, K. Thomas, R. Wilson, L. Hillier, R. Staden, N. Halloran, P. Green, J. Thierry-Mieg, L. Qiu, and S. Dear, Swiss-Prot accession number P30635.
Enzyme—Analysis of the oxidized form of TrxR (12) with non-reducing SDS-polyacrylamide gel electrophoresis showed that the enzyme migrated as a protein of $M_r$ 58,000 with no detectable species of higher molecular weight (not shown), demonstrating that in the oxidized enzyme, no intermolecular covalent bonds between the subunits were present (which would migrate at about 116,000 or higher in nonreducing SDS-polyacrylamide gel electrophoresis).

Cloning and Sequencing of a Rat TrxR cDNA Clone—The unique NH$_2$-terminal part of the bovine peptide containing the glutathione reductase active site motif (KVMVLDFVTPTPLGTRCG; see above) was used in database homology searches, which identified a rat EST cDNA clone as most likely encoding rat TrxR. This cDNA clone was isolated by expressed sequence tag analysis of differential gene expression upon treatment of neuroblastoma cells with nerve growth factor (31) and had been only partially sequenced.

Analysis of the rat cDNA clone revealed a size of the insert of more than 2 kilobase pairs, of which we subsequently determined the full sequence. The entire rat clone was 2193 nucleotides long and contained an open reading frame of 1497 nucleotides and 568-nucleotide-long 3′-untranslated region ending with an 18-nucleotide-long poly(A) tail. The rat cDNA sequence, except for the first 59 nucleotides, showed a high homology to the published putative human TrxR cDNA sequence (26). Nucleotides corresponding roughly to nucleotides 1–370 and 2505–3800 in the human sequence were missing in the rat cDNA clone, explaining its smaller size, while the full open reading frame and the part of the 3′-UTR containing a SECIS element (see below) was intact in the rat cDNA clone (Fig. 4).

The bovine peptide sequence RSGGNILQTG with ex-
experimental confirmation of X as selenocysteine (see above) corresponded to the COOH-terminal end of the translated open reading frame of the rat or human cDNA clones using TGA as a codon for incorporation of selenocysteine. The mechanisms for incorporation of selenocysteine in mammalian proteins are not yet fully clarified. However, it is clear that a TGA codon is not sufficient genetic information for encoding SeCys, but in addition, selenocysteine insertion sequences (SECIS elements) in the 3′-UTR of several mammalian selenoprotein mRNAs have been identified (34–39). In two recent reports (39, 40), consensus sequences and folding patterns of mammalian SECIS elements were proposed. We therefore examined the 3′-UTR of the human and rat thioredoxin reductase cDNA clone sequences and a typical SECIS element could be identified in both sequences, at approximately the same distance from the TGA codon (large box in Fig. 4). Although somewhat different from each other, the SECIS elements of both rat and human TrxR could form potential stem-loop structures, which agreed well with the eukaryotic SECIS folding structures proposed by both Low and Berry (39) and Walczak et al. (40). This is shown

FIG. 4. Nucleotide sequence alignment of rat and human cDNA clones. The sequence of the rat TrxR cDNA clone (lower sequence, this work) is aligned with that of the previously published (26) human TrxR cDNA clone (upper sequence). Both nucleotide sequences contain an open reading frame starting with ATG and ending with TGAGGTGAAA (indicated by small boxes in the alignment). In the 3′-untranslated region, potential SECIS elements (40) could be identified in both sequences (large box), shown in further detail in Fig. 5. Identical nucleotides between the two sequences are indicated by dots. The 5′- and 3′-ends of the human cDNA sequence are not shown.

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FIG. 5. Proposed SECIS elements of rat and human TrxR. Shown in A is a potential secondary structure of the 3'-UTR of the rat TrxR nucleotide sequence, as predicted using the FOLD computer program (Genetics Computer Group). The region forming a probable SECIS element is indicated, and nucleotides corresponding to this region are boxed in the alignment of the human and rat TrxR cDNA sequences (large box in Fig. 4). Using manual folding, the probable SECIS elements of both rat and human TrxR could be folded to secondary structures in close resemblance to the consensus of eukaryotic SECIS elements proposed by Walczak et al. (40) (B), as well as the eukaryotic form II consensus SECIS element proposed by Low and Berry (39) (C). In the upper part of panels B and C, the basic features of the consensus SECIS elements are given, with conserved and essential nucleotides for SECIS function shown in **boldface type**, including the adenines in the loop or bulge, and the “quartet” or “base of stem” motif comprising non-Watson-Crick base pairing. The manually folded rat and human TrxR SECIS elements are shown below the schematic structures of the consensus SECIS elements, with starting and ending nucleotide numbers indicated corresponding to the cDNA clones (see large box in Fig. 4).

**Homologies between Mammalian TrxR Amino Acid Sequences and Consensus with Other Members of the Enzyme Family**—Fig. 6 shows that the amino acid sequences determined from the bovine TrxR peptides had high homology to the translated open reading frames of the rat and human TrxR...
cDNA clone sequences, thereby conclusively confirming the identity of these clones. Due to the confirmation of SeCys in the carboxyl terminus of the bovine enzyme (Fig. 3) as well as the finding of SECIS elements in the rat and human TrxR cDNA sequences (Fig. 5), TGA is proposed to translate as a SeCys residue in Fig. 6. Shown in Fig. 6 is also the high homology between the mammalian TrxR sequences and human glutathione reductase.

The mammalian thioredoxin reductases contained all of the features of conserved structure domains of glutathione reductase, i.e. FAD, pyridine nucleotide, central domain, and interface domain (1). The highest homology to glutathione reductase (indicated by * or ♠ in Fig. 6) was in the interface region (i.e. within the last 150 amino acids) that governs the association of the two subunits in the dimeric holoenzyme. In alignments of glutathione reductase, lipoamide dehydrogenase, trypanothione reductase, and mercuric reductase, this is the region that displays the highest sequence conservation among these enzymes (1), which thereby holds true for mammalian thioredoxin reductase as well. Williams (1) has indicated which residues are identical among human glutathione reductase and the mammalian thioredoxin reductases and indicates with open triangles those amino acids in the first 300 amino acids in glutathione reductase that are conserved in the consensus sequences of the glutathione reductase family. The initial three amino acids of the rat TrxR shown in italics indicate a potential N-glycosylation motif. The boldface U denotes the selenocysteine residue.
idues among the first 300 amino acids in glutathione reductase that are conserved within the whole enzyme family. Out of these 36 conserved residues (Fig. 6, open triangles), only two were not conserved in the mammalian TrxR sequences (Ala\textsuperscript{130} and Ser\textsuperscript{295} in the rat enzyme). One interesting structural difference between the human and the rat predicted proteins is the consensus sequence for N-linked glycosylation (Asn-Asp-Ser) in the rat enzyme (Fig. 6). The significance of this is not known.

**DISCUSSION**

In this study, we found that a selenocysteine containing COOH-terminal motif (-Gly-Cys-SeCys-Gly-COOH) is conserved among rat, calf, and human TrxR and that both rat and human cDNA sequences carry consensus sequences in the 3'-UTR necessary for the proper incorporation of selenocysteine during translation of the mRNA. We have also shown that the carboxyl-terminal selenocysteine and cysteine residues are important for the catalytic activity of the enzyme.

TrxR from *E. coli* is by far the best characterized thioredoxin reductase. Differences between this enzyme and other pyridine nucleotide-disulfide oxidoreductases, such as glutathione reductase or lipoamide dehydrogenase, have been noted and discussed for quite some time, regarding differences in molecular weight, substrate specificities, and their primary and three-dimensional structures (1, 11, 12). The sequences presented here of both bovine TrxR peptides and a rat TrxR cDNA clone confirm the putative human TrxR cDNA sequence presented earlier (26), which so far has been the only mammalian TrxR sequence described.\(^4\) Clearly, mammalian TrxR, in contrast to *E. coli* TrxR, is structurally closely related to glutathione reductase and other members of this enzyme family. The motif in the N\textsubscript{H}\textsubscript{1}-terminal part of the enzyme containing the active site disulfide (Cys-Val-Asn-Val-Gly-Cys) is conserved, as well as the FAD domain and the NADPH binding regions, and there is a preservation of key residues important for the catalytic function (Fig. 6). In good agreement with this, a recent study of Williams and co-workers (32) using spectroscopic methods demonstrated the clear similarities in mechanism between human TrxR and glutathione reductase or lipoamide dehydrogenase, distinct from that of TrxR from *E. coli*. However, the selenocysteine-containing COOH-terminal part of mammalian TrxR is an addition to the basic structure that should play a significant role for the catalysis of TrxR distinct from that of glutathione reductase or lipoamide dehydrogenase, which lack this selenocysteine containing COOH-terminal elongation.

We found that the atomic absorption of TrxR purified from normal mammalian sources had a content of 0.6 selenium atom/58-kDa subunit (27), while Tamura and Stadtman (25) reported that TrxR from a human T cell line contained 0.74 atom/58-kDa subunit (27), while Tamura and Stadtman (25) reported that TrxR from a human T cell line contained 0.74 atom/58-kDa subunit (27), while Tamura and Stadtman (25) reported that TrxR from a human T cell line contained 0.74 atom/58-kDa subunit (27). However, the selenocysteine-containing COOH-terminal part of mammalian TrxR is an addition to the basic structure that should play a significant role for the catalysis of TrxR distinct from that of glutathione reductase or lipoamide dehydrogenase, which lack this selenocysteine containing COOH-terminal elongation.

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We found that the atomic absorption of TrxR purified from normal mammalian sources had a content of 0.6 selenium atom/58-kDa subunit (27), while Tamura and Stadtman (25) reported that TrxR from a human T cell line contained 0.74 atom/58-kDa subunit (27). However, the selenocysteine-containing COOH-terminal part of mammalian TrxR is an addition to the basic structure that should play a significant role for the catalysis of TrxR distinct from that of glutathione reductase or lipoamide dehydrogenase, which lack this selenocysteine containing COOH-terminal elongation. The reason for the resistance to cleavage of TrxR in its oxidized state could be that the COOH-terminal part is sterically protected in the oxidized enzyme but not in the reduced form. Also, analysis of calf thymus or rat liver TrxR with nonreducing SDS gel electrophoresis revealed no species with higher apparent molecular mass than 58 kDa, ruling out the possibility of intermolecular covalent bonds in the oxidized dimeric holoenzyme, that could otherwise possibly include the Cys or SeCys residues at the COOH-terminal end (and also affect cleavage with carboxypeptidase). However, the most likely explanation is that the SeCys residue forms a redox active bridge with the neighboring Cys residue, as also discussed by both Williams and Bovine Thioredoxin Reductase Structure and Function 8589
and co-workers (32) and Stadtman and co-workers (44). When such a Cys-SeCys bridge is present in the oxidized form of the enzyme, next to the COOH-terminal Gly residue, this is not a substrate for carboxypeptidase-catalyzed cleavage. It has long been known that the nature of the residues at the NH2-terminal side of the most carboxyl-terminal residue to be cleaved by carboxypeptidases may affect the affinity of the protease for its substrate (45). In this context, it should also be noted that inactivation of TrxR with low molecular weight electrophilic compounds like 1-chloro-2,4-dinitrobenzene (23) or 1,3-bis-(2-chloroethyl)-1-nitrosourea (32) is fast in the presence of NADPH but negligible in the absence of NADPH. These observations favor the notion of a Cys-SeCys bridge in the oxidized enzyme (nonaccessible for alkylation), provided that these postulated redox active residues in their reduced state indeed are such a Cys-SeCys bridge is present in the oxidized form of the enzyme.

Furthermore, it should be commented that in the amino acid analysis of residues liberated upon carboxypeptidase Y treatment of reduced TrxR, Gly and SeCys could be identified by analysis of residues liberated upon carboxypeptidase Y treatment. It has long been known that the nature of the residues at the NH2-terminal side of the most carboxyl-terminal residue to be cleaved by carboxypeptidases may affect the affinity of the protease for its substrate (45). In this context, it should also be noted that inactivation of TrxR with low molecular weight electrophilic compounds like 1-chloro-2,4-dinitrobenzene (23) or 1,3-bis-(2-chloroethyl)-1-nitrosourea (32) is fast in the presence of NADPH but negligible in the absence of NADPH. These observations favor the notion of a Cys-SeCys bridge in the oxidized enzyme (nonaccessible for alkylation), provided that these postulated redox active residues in their reduced state indeed are such a Cys-SeCys bridge is present in the oxidized form of the enzyme.

Interestingly, Trx reduction was impaired to a greater extent than DTNB reduction upon treatment of TrxR with carboxypeptidase Y (Fig. 2B). A possible explanation for this difference could be that DTNB may be reduced directly by the glutathione reductase-like active site disulfide in the NH2-terminal part of the enzyme, in addition to reduction via the selenocysteine-containing COOH-terminal redox active site, whereas Trx only can be reduced by the latter.

The COOH-terminal elongation may possibly explain the wide substrate specificity of mammalian TrxR compared with glutathione reductase or lipoamide dehydrogenase, suggesting that the redox substrate Cys-SeCys couple at the COOH-terminal end is highly accessible when reduced. With the overall structure as well as the mechanism otherwise being similar to glutathione reductase, it may be proposed that the NH2-terminal positioned redox-active disulfide of one subunit in the dimeric holoenzyme is reduced by NADPH via the flavin of the same polypeptide chain, thereafter interacting with the COOH-terminal Cys-SeCys couple of the other subunit. The interface region that governs the association of the two subunits in the glutathione reductase enzyme family is highly conserved also in TrxR, and the dimeric holoenzymes of this enzyme family all associate with the subunits inverted with respect to each other, so that the NH2-terminal end of one subunit interacts with the COOH terminus of the other (1). The additional redox-active disulfide at the COOH-terminal end of mercuric reductase (1, 46) is highly analogous to the proposed Cys-SeCys redox active couple in TrxR. Interestingly, two genes of Plasmodium falciparum were recently identified to encode for a glutathione reductase (47) and a thioredoxin reductase (48); and while both are homologous in sequence to human glutathione reductase and TrxR (49), only the thioredoxin reductase has a COOH-terminal elongation. Although this seems to contain no SeCys residue, a potential redox-active Cys-Gly-Gly-Gly-Lys-Cys motif is found in this elongated part of the enzyme. Moreover, in database searches, two different genes of Caenorhabditis elegans are found, both highly homologous to mammalian TrxR but one with a Gly-Cys-Gly motif at the COOH terminus.
and one\(^5\) with an open reading frame that could possibly translate into Gly-Cys-SeCys-Gly at the COOH terminus, provided that TGA would translate as SeCys and TAA terminating translation (as in the case of mammalian TrxR). It is not certain that these genes actually are translated in this manner, but if so, \(C.\) \textit{elegans} would thereby have two proteins similar to mammalian TrxR, both with potential redox active motifs at the COOH terminus but one with a Cys-Cys couple and one with a Cys-SeCys couple. The COOH-terminal ends of these proteins are aligned in Table II. The function and importance of the potential redox-active residues indicated certainly deserves further study.

Unlike human TrxR, the rat enzyme contained a potential asparagine-linked oligosaccharide attachment site, Asn\(^2\)-Asp\(^3\)-Ser\(^4\). The corresponding human sequence was Asn 2-Gly3-Pro4, U61947 (gene \textit{C06G3}zyme and its inhibition by some clinically used drugs. The larger size is due to a COOH-terminal elongation in the human enzyme was earlier reported to reveal a sequence starting two residues COOH-terminally of the proposed initiating methionine (26). The explanation of these inconclusive findings regarding the NH\(_2\) terminus of the enzyme is not established. The results may indicate a blocked NH\(_2\) terminus, which possibly is easily degraded during purification. Alternatively, the results may reflect NH\(_2\)-terminal heterogeneity of the enzyme, purified from different sources. Attached carbohydrates and NH\(_2\)-terminal heterogeneities could perhaps explain some of the differences in estimations of molecular mass of mammalian TrxR, reported to be 54,171 Da (predicted) (26), 55 kDa (24), 57 kDa (25), 58 kDa (12), or 60 kDa (13). Interestingly, COS cells transfected with a human TrxR cDNA construct\(^4\) produced three different recombinant proteins with molecular masses of \(~55–60\) kDa, detected using polyclonal antibodies and Western blotting, while nontransfected lung adencarcinoma cells only contained a 55-kDa protein, detected using the same antibodies (33). The larger proteins in the transfected COS cells would thereby have two proteins similar to \textit{C.\) elegans\(\text{TrxR}\). Further study is needed to determine the potential redox-active residues indicated certainly deserves further study.

In conclusion, mammalian TrxR is a recently discovered selenoprotein with a structure closely related to glutathione reductase. The larger size is due to a COOH-terminal elongation with a conserved selenocysteine-containing motif (Gly-Cys-SeCys-Gly), which we show is redox-active and which may explain the exceptionally wide substrate specificity of the enzyme and its inhibition by some clinically used drugs.

Acknowledgments—The help of Lena Hernberg with amino acid sequencing, Carina Palmberg with determination of amino acid composition, and Dr. Yuanli with initial predictions of secondary structural motifs is greatly acknowledged. We also acknowledge helpful discussions with Drs. Yair Aharowitz, Thressa Stadtman, and Charles Williams, Jr.

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