Molecular Cloning and Characterization of a Mitochondrial Selenocysteine-containing Thioredoxin Reductase from Rat Liver*

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A thioredoxin reductase (TrxR), named here TrxR2, that did not react with antibodies to the previously identified TrxR (now named TrxR1) was purified from rat liver. Like TrxR1, TrxR2 was a dimeric enzyme containing selenocysteine (Secys) as the COOH-terminal penultimate residue. A cDNA encoding TrxR2 was cloned from rat liver; the open reading frame predicts a polypeptide of 526 amino acids with a COOH-terminal Gly-Cys-Secys-Gly motif provided that an in-frame TGA codon encodes Secys. The 3′-untranslated region of the cDNA contains a canonical Secys insertion sequence element. The deduced amino acid sequence of TrxR2 shows 54% identity to that of TrxR1 and contained 36 additional residues upstream of the experimentally determined NH2-terminal sequence. The sequence of this 36-residue region is typical of that of a mitochondrial leader peptide. Immunoblot analysis confirmed that TrxR2 is localized almost exclusively in mitochondria, whereas TrxR1 is a cytosolic protein. Unlike TrxR1, which was expressed at a level of 0.6 to 1.6 μg/milligram of total soluble protein in all rat tissues examined, TrxR2 was relatively abundant (0.3 to 0.6 μg/mg) only in liver, kidney, adrenal gland, and heart. The specific localization of TrxR2 in mitochondria, together with the previous identification of mitochondria-specific thioredoxin and thioredoxin-dependent peroxidase, suggest that these three proteins provide a primary line of defense against H2O2 produced by the mitochondrial respiratory chain.

Thioredoxin (Trx) is a widely expressed 12-kDa protein that performs pleiotropic cellular functions (1, 2). The active site of Trx contains the sequence -Cys-Gly-Pro-Cys-, and the reduced form of the protein serves as a hydrogen donor for ribonucleotide reductase (3), protein methionine sulfoxide reductase (4), thioredoxin-dependent peroxidase (5), and protein tyrosine phosphatase (6) as well as contributes to the up-regulation of various transcription factors (7–11). In addition, the reduced form, but not the oxidized form, of Trx binds to and inhibits the catalytic activity of apoptosis signal-regulating kinase, also known as mitogen-activated protein kinase kinase kinase (12). Furthermore, Trx serves as a growth factor that stimulates the proliferation of T lymphocytes (13).

Oxidized Trx is converted back to the reduced form by thioredoxin reductase (TrxR) with the use of electrons from NADPH (14, 15). TrxR is a homodimeric enzyme with a redox-active disulfide and contains one FAD molecule per subunit (14, 15). It belongs to a superfamily of flavoprotein disulfide oxidoreductases that includes glutathione reductase (GR), dihydrolipoamide reductase, mercuric reductase, and alkylhydroperoxide reductase (16, 17). Mammalian TrxR is distinct from those of prokaryotes and yeast. The mammalian enzyme exhibits a broader substrate specificity, having the ability to reduce chemically unrelated compounds such as selenite and 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of NADPH (18, 19), is larger in subunit size (58 kDa, compared with 35 kDa for prokaryote and yeast enzymes), and contains a much longer COOH-terminal region (18, 20). In addition, mammalian TrxR is a selenoprotein that contains a penultimate selenocysteine (Secys) residue in the sequence -Gly-Cys-Secys-Gly (14, 21–23), which can serve as a redox center (24).

Mammalian cells contain two distinct forms of Trx: Trx1 is located in the cytosol and nucleus and is also secreted (10), whereas Trx2 is restricted to mitochondria (25). However, only one isoform of TrxR has previously been identified in mammalian cells, and it has not been known whether this protein is expressed in mitochondria. We now describe the purification and cloning of a second type of TrxR, named TrxR2, from rat liver and demonstrate that this protein is specifically expressed in mitochondria.

**EXPERIMENTAL PROCEDURES**

Materials—Rat liver was obtained from Bioproducts for Science, Inc. (Indianapolis, IN). Rabbit antiserum to rat TrxR1 was produced by immunization with purified enzyme according to standard procedures. Rabbit antiserum to TrxR2 was prepared by injection with a hemocyanin-conjugated peptide (RSGLDPTVTGCCG), corresponding to the COOH-terminal sequence of rat TrxR2, with the exception that the penultimate residue (Secys) was changed to Cys. Horseradish peroxidase-conjugated antibodies to rabbit immunoglobulin G and the enhanced chemiluminescence (ECL) immunoblot detection system were from Amersham; biotin-conjugated iodoacetamide, N-(iodoacetyl)ethylenediamine (BIAM), was from Molecular Probes; horseradish peroxidase-conjugated streptavidin, 3,3',5,5'-tetramethyl benzidine, and Neutravidin beads were from Pierce; and yeast GR was from Boehringer-Mannheim. Recombinant rat Trx was prepared as described (26).
Purification of TrxR1—Rat livers (1 kg) were homogenized in 4 liters of 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.05 mM 4-(3-aminomethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), peptideatin (0.5 μg/mL), leupeptin (0.5 μg/mL), and apronin (0.5 μg/mL). The homogenate was centrifuged at 70,000 × g for 30 min. The supernatant was then eluted to a DEAE-Sephadex column (10 × 16 cm) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 0.5 M NaCl. The column was washed with 1.5 liters of equilibration buffer containing 20 mM NaCl and 200 mM KCl. The remaining three-fourths of the BIAM-labeled TrxR2 digest were eluted with a linear gradient of 0 to 60%, v/v, of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min over 60 min. Fractions (500 μL) were collected, and a portion (2 μL) of each was analyzed for BIA-labeled peptide. Peptides were further purified by HPLC on a C18 column as described above, and the BIAM-labeled peptide that eluted at 28.5 min was also collected and subjected to sequence analysis.

Purification of TrxR2—The pellet derived from the pH 5 precipitation step described for the purification of TrxR1 was dissolved in 2 liters of 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 0.05 mM AEBSF, leupeptin (0.5 μg/mL), and apronin (0.5 μg/mL). The pH of the suspension was adjusted to 7.8 with 1 M ammonium hydroxide. After centrifugation of the suspension, the supernatant (40 g of protein) was applied to a Phenyl-SPW high-performance liquid chromatography (HPLC) column (0.75 × 7.5 cm) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 0.01 mM AEBSF. The column was washed with 100 mM NaCl in 5 liters of equilibration buffer, and fractions (25 mL) were collected and assayed for TrxR1 by immunoblot analysis.

The peak fractions (10.4 g of protein), corresponding to 300 to 380 mM NaCl on the gradient, were pooled, dialyzed overnight against 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 0.01 mM AEBSF. The resulting supernatant was dehydrated to 2.5 liters of equilibration buffer and then eluted with 2.5 liters of equilibration buffer, 2.5 liters of 1 M DTT, and 0.01 mM AEBSF, leupeptin (0.5 μg/mL), and apronin (0.5 μg/mL). The pH of the suspension was adjusted to 7.8 with 1 M ammonium hydroxide. After centrifugation of the suspension, the supernatant (40 g of protein) was applied to a Phenyl-SPW high-performance liquid chromatography (HPLC) column (0.75 × 7.5 cm) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 0.01 mM AEBSF. The column was washed with 100 mL of equilibration buffer, and proteins were then eluted stepwise with 100 mL each of equilibration buffer containing 200 mM KCl, equilibration buffer containing 200 mM sodium phosphate and 200 mM KCl, and equilibration buffer containing 1 mM NaCl and 200 mM KCl. The 58-kDa TrxR1 was present almost exclusively in the fractions eluted by the buffer containing 200 mM KCl as revealed both by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining and by immunoblot analysis.

The purity and mass of the isolated peptides were assessed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF) mass spectrometry (Hewlett-Packard model G1005A, using sinapinic acid as the matrix (21)). Electrospray mass spectrometry was performed with a Hewlett-Packard model G1945A instrument interfaced to a model 1100 HPLC system equipped with a Vydac 218TP narrow bore C4 column. The effluent from the column (200 μL/min) was mixed in a tee with a 1 mM acetic acid, pumped by another 1100 series pump (100 μL/min), and the mixture was introduced into the mass spectrometer (27). Sequences were determined by automated Edman degradation with a Hewlett-Packard model G1005A sequencer running version 3.5 of the manufacturer’s chemistry program.

After removal of the resulting precipitate by centrifugation, the supernatant (61.5 mg of protein) was applied to a Phenyl-SPW HPLC column (0.75 × 7.5 cm) that had been equilibrated with 20 mM Hepes-NaOH (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 1.2 M ammonium sulfate. The column was washed with 60 mL of equilibration buffer, and proteins were then eluted with a decreasing linear gradient of 0 to 0.8 to 0.64 M ammonium sulfate on the gradient, were pooled, dialyzed overnight against equilibration buffer containing 200 mM KCl and 200 mM NaCl containing 100 mM NaCl. Proteins were eluted between 32 and 42 min (2.1 mg of protein) were pooled, concentrated, dialyzed against 20 mM Hepes-NaOH (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 1 mM sodium phosphate, and then applied to a 21-cm column (21) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. The column was washed with 100 mL of equilibration buffer containing 200 mM NaCl and 200 mM KCl. Fractions were assayed for TrxR2 by immunoblot analysis (see Fig. 6D). Peak fractions eluted between 32 and 42 min (2.1 mg of protein) were pooled, concentrated, dialyzed against 20 mM Hepes-NaOH (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 1 mM sodium phosphate, and then applied to a 21-cm column (21) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. Fractions (2 mL) were collected and assayed for TrxR2 by immunoblot analysis (see Fig. 6D). Peak fractions eluted between 32 and 42 min (2.1 mg of protein) were pooled, concentrated, dialyzed against 20 mM Hepes-NaOH (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 1 mM sodium phosphate, and then applied to a 21-cm column (21) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. Fractions (2 mL) were collected and assayed for TrxR2 by immunoblot analysis (see Fig. 6D). Peak fractions eluted between 32 and 42 min (2.1 mg of protein) were pooled, concentrated, dialyzed against 20 mM Hepes-NaOH (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 1 mM sodium phosphate, and then applied to a 21-cm column (21) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. Fractions (2 mL) were collected and assayed for TrxR2 by immunoblot analysis (see Fig. 6D). Peak fractions eluted between 32 and 42 min (2.1 mg of protein) were pooled, concentrated, dialyzed against 20 mM Hepes-NaOH (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 1 mM sodium phosphate, and then applied to a 21-cm column (21) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. Fractions (2 mL) were collected and assayed for TrxR2 by immunoblot analysis (see Fig. 6D). Peak fractions eluted between 32 and 42 min (2.1 mg of protein) were pooled, concentrated, dialyzed against 20 mM Hepes-NaOH (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 1 mM sodium phosphate, and then applied to a 21-cm column (21) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. Fractions (2 mL) were collected and assayed for TrxR2 by immunoblot analysis (see Fig. 6D). Peak fractions eluted between 32 and 42 min (2.1 mg of protein) were pooled, concentrated, dialyzed against 20 mM Hepes-NaOH (pH 7.5) containing 1 mM EDTA, 1 mM DTT, and 1 mM sodium phosphate, and then applied to a 21-cm column (21) that had been equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. Fractions (2 mL) were collected and assayed for TrxR2 by immunoblot analysis (see Fig. 6D).
was calculated as micromoles of NADPH oxidized per minute at 25 °C from the relation $A_{340} \times 0.56/6.22$. Assay mixtures lacking enzyme served as controls.

Cloning and Sequencing of TrxR2 cDNA from a Rat Liver cDNA Library—Complementary DNA encoding TrxR2 was amplified from Marathon-ready rat liver cDNA (CLONTECH) by the polymerase chain reaction (PCR) with the 5′ primer 5′-CA/GA/CAG/GAA/AC/C/T/T/T/GA/TGA and 3′ primer 5′-GTNACNGCTGCGTGGAGG, corresponding to the determined NH₂-terminal (QQNFDDLIVIIGGS) and COOH-terminal SGLDPTVYGCCG (where U represents Secys) sequences, respectively, of the purified protein. The PCR products were separated on a 1% agarose gel, and the amplified 1.5-kilobase molecule was eluted from the gel with a Qiagen gel extraction kit. After ligation of the eluted DNA into the pCR3.1 vector (Invitrogen) and transformation of Escherichia coli, positive clones were identified by nested-PCR with the internal forward primer 5′-GA/C/T/GA/CT/AT/NT/CT/GGG or the internal reverse primer 5′-CCA/G/A/AA/A/AN/GA/TG/C/G/A/T/CT, both of which were derived from the internal amino acid sequence HGITSDDIFWLK of TrxR2. Plasmid DNA was purified from the positive E. coli clones with a Qiagen miniplasmid preparation kit, and was sequenced with the T7 primer and pCR3.1 reverse primer on an ABI sequencer. To extend the 5′ sequence, we performed 5′-rapid amplification of cDNA ends (5′-RACE) by PCR with Marathon-Ready rat liver cDNA as the template, the adapter primer 1 (CLONTECH) as the forward primer, and a reverse primer complementary to the sequence 5′-CTGGCTGCTAGATGATGGA. 5′-RACE was also performed with nested adapter primer 2 (CLONTECH) as the forward primer and a reverse primer complementary to the sequence 5′-GCAGGAGAACCTGATCTC. PCR products were cloned into the TA vector and sequenced. The 5′-extended sequences determined from the two independent 5′-RACE experiments were identical. Similarly, the sequence of the 3′-translated region was determined by two independent 3′-RACE experiments with two different sense primers (5′-GCTCTAGAGCCAGGTGTAGCAG and 5′-TGTTAAGAGCTCCACATGCTC) and the adapter primer 1 as the antisense primer.

Analytical Ultracentrifugation—The Beckman Optima model XL-A analytical ultracentrifuge equipped with a four-place An-Ti rotor was used for sedimentation velocity experiments at 20.0 °C. The density ($\rho$) of the dialysate buffer (10 mM sodium phosphate-1.8 mM potassium phosphate (pH 7.4), 137 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (substituted for 1 mM DTT in centrifugation studies)) was determined to be 1.00546 g/ml at 20.0 °C ± 0.1 °C with a Paar DMA 58 densitometer, and the relative viscosity was determined to be 1.020 (28). The partial specific volumes ($\upsilon$) of TrxR1 and TrxR2 were calculated to be 0.720 and 0.722 ml/g (29), respectively, from the amino acid sequences. The protein sample (0.34 ml) and dialysate buffer (0.35 ml) were loaded into the right and left sides, respectively, of a 4° Kel-F coated double-sector centerpiece in 12-mm cells that were equipped with a quartz window for ultracentrifugation velocity calibration. Experiments were performed at 48,000 and 40,000 rpm for TrxR1 and TrxR2, respectively, while scanning in a continuous mode (0.003-cm steps) with triple averaging at 280 nm and 4-min intervals (after equilibration and radial calibration at 3000 rpm, at which speed radial and wavelength (9 to 11 averages at 1-nm resolution) scans were collected). The TRACKER program of A. P. Minton (http://bbri-wwww.eei.harvard.edu/RASMB/ rasmh.html) was used to monitor the progress of runs. Observed sedimentation coefficients ($s_{20,w}$) were corrected to the density and viscosity of water at 20.0 °C, yielding $s_{20,w}$ values of 1.0393 and 1.0395 for TrxR1 and TrxR2, respectively. The time derivative method of Stafford (30, 31) was used to estimate the molecular weights of TrxR1 and TrxR2, the diffusion coefficient ($D$) and sedimentation coefficient ($s$) were obtained from the half-width and maximum, respectively, of the Gaussian fit to the $g(s)$ distribution pattern from four late scans (Origin Windows g(s) Velocity Program of Beckman Instruments), and the solute molecular weight ($M_\text{solute}$) was calculated from the Svedberg equation, $M = RTs/[D(1 - \upsilon)]$. The relation of $D$ to the half-width or standard deviation of the Gaussian fit is given by $D = (r_m a^2 t^2 I)$, where $t$ is the sedimentation time in seconds, $a^2$ is the angular velocity of the rotor, and $r_m$ is the radial position of the meniscus (30, 31); $I$ is the intensity of the scan, and $g(s)$ can be read from the output file of Beckman g(s) program.

Atomic Absorption Spectrometry—The selenium content of TrxR1, TrxR2, and the BIAM-labeled peptide derived from BIAM-labeled TrxR2 was determined with a Perkin-Elmer model 4100 ZL atomic absorption spectrometer with the use of a palladium-magnesium nitrate modifier and temperature conditions as described (32). Calibration solutions were prepared by diluting the selenium stock solution (1 g/liter) with a solution containing 1.16 mM Na₂HPO₄, 0.31 mM KH₂PO₄, 10.26 μM NADPH, and bovine serum albumin (0.120 mg/ml) to give final concentrations of 0, 10, 30, and 90 μg/liter. Various concentrations of TrxR1 and TrxR2 were prepared in the same solution devoid of albumin; the BIAM-labeled peptide solution was prepared in double-distilled water.

Distribution of TrxR Isozymes in Rat Tissues and in Subcellular Fractions of Rat Liver—Frozen rat tissues (Pel-Freeze Biologicals) were sonicated in a solution containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, aprotinin (2.5 μg/ml), and leupeptin (5 μg/ml). The sonicates were centrifuged at 100,000 × g × 15 min, and the resulting supernatants were subjected to immunoblot analysis with antibodies specific for TrxR1 or TrxR2. Rat liver homogenates were prepared, and cytosolic and mitochondrial fractions were separated by ultracentrifugation as described (33, 34).
Fig. 2. Purification of tryptic peptides derived from BIAM-labeled TrxR2. A, TrxR2 was labeled with BIAM and digested with trypsin, and the resulting peptides were analyzed on a C18 column, with elution monitored on the basis of A215. The positions of BIAM-labeled peptide a and of two other peptides, b and c, that were subjected to sequence determination are indicated. B, each fraction from the C18 column in A was assayed for BIAM-labeled peptides with the use of horseradish peroxidase-conjugated streptavidin and the peroxidase substrate 3,3',5,5'-tetramethyl benzidine, the oxidation of which was monitored spectrophotometrically at 405 nm. C, the tryptic digest of BIAM-labeled TrxR2 was subjected to affinity purification with Neutravidin beads, and the resulting purified peptides were analyzed on a C18 column as described in A. The position of BIAM-labeled peptide a is indicated.
FIG. 3. Nucleotide sequence of rat liver TrxR2 cDNA and the deduced amino acid sequence of the encoded protein. The ATG codon encoding the initiating methionine, the TGA triplet encoding Sec (U), and the termination codon TAA are indicated by bold type. The experimentally determined NH₂-terminal sequence of purified rat liver TrxR2, as well as the sequences of two internal tryptic peptides and of the BIAM-labeled peptide, are underlined. Nucleotide sequences used for RACE experiments are also underlined. Double underlines indicate the putative mitochondrial targeting sequence in the predicted protein and the nucleotide sequences of the three segments conserved in the SECIS element. Nucleotide residue numbers are indicated on the left. The nucleotide sequence of rat TrxR2 has been deposited in GenBank under the accession number AF072865.
was purified with Neutravidin affinity matrix and a C18 column. Protein was cleaved with trypsin, and a BIAM-labeled peptide HGITSDDIFWLK. In addition, TrxR2 was subjected to autolysis and yielded the sequences IIVDAQEATSVPHIYAIGDV and SGLDPTVTGCX.

Edman degradation of the labeled peptide yielded the sequence deduced from the cDNA, and confirming the amino-terminal site as well as the presence of selenocysteine.

TrxR1 was similarly labeled with BIAM, and the BIAM-labeled peptide generated by digestion with endoproteinase Lys-C was purified. Sequencing and MALDI-TOF analysis of the labeled peptide (data not shown) yielded the sequence RSGGDILQSGCUG (where U represents Secys), which matches exactly the sequence of amino acids 486 to 498 at the COOH terminus of the previously identified TrxR from rat liver (18).

Cloning and Sequencing of TrxR2 cDNA—TrxR2 cDNA was amplified from a rat liver cDNA library by PCR with primers based on the NH2- and COOH-terminal amino acid sequences of the purified protein, as described under “Experimental Procedures.” A 1.5-kilobase PCR product was obtained, the cloning and sequencing of which revealed a 1463-base pair fragment that contained the precise coding sequences in the same reading frame for the two internal tryptic peptides derived from purified TrxR2 (Fig. 3). Additional 5’ and 3’ sequences were obtained with the use of RACE-PCR, yielding a cumulative sequence of 1982 base pairs, excluding the poly(A) tail (Fig. 3).

The translational initiation site was assumed to be the methionine codon composed of nucleotides 29 to 31, which was the first ATG triplet downstream of an in-frame nonsense codon (TAA at nucleotides 14 to 16). Two translational termination codons, TGA and TAA, occurred in-frame in the sequence TGGAGTTAA (nucleotides 1601 to 1609). As in other Secys-containing proteins, the TGA codon corresponds to the penultimate Secys residue. Therefore, the TAA triplet was assumed to be the termination codon. The open reading frame encodes a polypeptide of 526 amino acids, with a calculated molecular mass of 56,574.8 daltons.

The deduced protein sequence contained 36 residues upstream of the experimentally determined NH2-terminus of purified TrxR2. This additional 36-residue sequence contains 6 arginine residues and no acidic residues, and it is predicted to form an α-helical structure (Plotstructure program of the University of Wisconsin Genetics Computer Group). The predicted high isoelectric point and α-helical structure are hallmarks of most mitochondrial leader peptides (36). Furthermore, like many mitochondrial precursor proteins, the predicted TrxR2 protein contains an arginine residue at position −10 (relative to the NH2-terminal residue of the mature protein) (37). Therefore, the mature TrxR2 comprises 490 amino acids, with a calculated molecular mass of 53,036 daltons; for comparison, the calculated molecular mass of the 498-residue rat TrxR1 is 54,491 daltons (18). The minor NH2-terminal sequence GGGQNFDDLWIGGSGGLA.

RESULTS

Purification of TrxR1 and TrxR2—In previous studies, we have used the Trx system as the electron donor for reduction of H2O2 by peroxiredoxins (26) and for reactivation of H2O2-inactivated protein tyrosine phosphatase (6). For these experiments, the 58-kDa TrxR, designated here as TrxR1, was routinely purified from rat liver by a procedure that included acidification of tissue homogenate to pH 5 and sequential chromatography of the acid-soluble proteins on DEAE Sephacel, 2′,5′-ADP-agarose affinity matrix, and Phenyl-Sepharose.

A purification in which the pH 5 precipitation step was inadvertently omitted yielded two peaks of flavoprotein after the 2′,5′-ADP-agarose column step, as judged from the ratio of A280/A460. The first peak eluted with the buffer containing 200 mM KCl and contained a protein with an apparent molecular mass of 58-kDa, whereas the second peak eluted with the buffer containing 1 mM NaCl and 200 mM KCl and comprised predominantly a 55-kDa protein. Further purification of these peak fractions by phenyl-Sepharose column chromatography yielded highly purified preparations of the 58- and 55-kDa proteins (Fig. 1A). Whereas both preparations exhibited TrxR activity, polyclonal antibodies to 58-kDa TrxR1 recognized the 58-kDa protein from the first peak but not the 55-kDa protein from the second peak (Fig. 1B), suggesting that the 55-kDa protein was not derived from TrxR1. The 55-kDa protein was thus named TrxR2. Like TrxR1, TrxR2 was demonstrated to be a flavin-containing protein by its absorption spectrum, which showed maxima at 280, 352, and 446 nm in a ratio of 1, 0.12, and 0.11, respectively (Fig. 1C).

Sequences of Peptides Derived from TrxR2—TrxR1 contains a penultimate Secys residue at its COOH terminus that can be selectively labeled with an alkylating agent (35). To determine whether TrxR2 also contains such a residue, the purified protein was labeled with 10 μM BIAM at pH 6.5 and subsequently incubated with 2 μM iodoacetamide at pH 7.5. The labeled protein was cleaved with trypsin, and a BIAM-labeled peptide was purified with Neutravidin affinity matrix and a C18 column (Fig. 2). Edman degradation of the labeled peptide yielded the sequence SGLDPTVTGCX; the residue corresponding to cycle 10 was identified as carboxymethylated cysteine, and the residue corresponding to cycle 11 was unknown. Determination of the molecular mass of the purified peptide by MALDI-TOF mass spectrometry yielded a mass of 1540.8 (data not shown), which is in excellent agreement with the molecular mass of the purified peptide by MALDI-TOF mass spectrometry yielding a mass of 1540.8 (data not shown), with the major sequence being readable for 20 cycles, with the majority sequence for 19 cycles: GGQNFDDLWIGGSGGLA. A minor sequence was consistent with loss of the first glycine residue. Two preparations of TrxR2 were analyzed by electrospray mass spectrometry, giving a mass of 53,036 calculated from the sequence deduced from the cDNA, and confirming the amino-terminal site as well as the presence of selenocysteine.

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The deduced TrxR2 sequence shows 54% identity (62% similarity) to both rat (18) and human (20) TrxR1 sequences (Fig. 4). Rat TrxR proteins show a low sequence homology to prokaryote and yeast TrxR enzymes (28 and 36% identity to E. coli (16) and yeast (5) TrxR, respectively), and they are distinguished from these enzymes by the presence of a COOH-terminal extension containing the Secys residue. TrxR2 showed a relatively high homology to human GR (38) (41% identity and similarity).

Fig. 4. Alignment of the amino acid sequences of TrxR and GR proteins from various species. Dashes within sequences represent gaps introduced to optimize alignment. Residue numbers are shown on the right. The last two amino acids (underlined) of C. elegans TrxR were translated as Secys and Gly on the basis of the published nucleotide sequence (GenBank accession number, U61947).

The deduced TrxR2 sequence shows 54% identity (62% similarity) to both rat (18) and human (20) TrxR1 sequences (Fig. 4). Rat TrxR proteins show a low sequence homology to prokaryote and yeast TrxR enzymes (28 and 36% identity to E. coli (16) and yeast (5) TrxR, respectively), and they are distinguished from these enzymes by the presence of a COOH-terminal extension containing the Secys residue. TrxR2 showed a relatively high homology to human GR (38) (41% identity and similarity).
50% similarity) as well as to putative GR sequences of *Caenorhabditis elegans* (39) and *Drosophila melanogaster* (40) (48 to 50% identity, 57 to 58% similarity). Furthermore, unlike human and mouse GR proteins, the *C. elegans* and *D. melanogaster* enzymes possess long COOH-terminal regions that are similar to those of mammalian TrxR proteins. The COOH termini of *C. elegans* and *D. melanogaster* GR proteins also end with the sequences GCCG and SCCS, respectively, which resemble the GCUG motif at the COOH termini of mammalian TrxR enzymes. As a result of the effort to sequence *C. elegans* chromosome III (41), another putative GR gene (GenBank accession number, U61947) has been identified on the basis of its homology (36% identity and 45% similarity) to mammalian GR enzymes. However, the gene product has not been shown to possess GR activity, and, at the time the gene was characterized, mammalian TrxR genes had not been identified. Comparison of the predicted amino acid sequence of the protein encoded by the chromosome III gene with those of TrxR1 and TrxR2 revealed higher homology (48 to 59% identity and 58 to 69% similarity) to these proteins than to mammalian GR enzymes. Furthermore, in mammalian TrxR enzymes, the codons for Secys (TGA) and Gly (GGT) in the chromosome III gene are followed by the stop codon TAA, indicating that the gene product could contain the sequence GCUG at its COOH terminus. Therefore, we listed the product of the chromosome III gene as a TrxR rather than as a GR in Fig. 4. TrxR2 showed low homology to other members of the flavoprotein disulfide oxidoreductase family, such as human dihydrolipoamide reductase (42) (28% identity) and *Pseudomonas aeruginosa* mercuric reductase (43) (26% identity).

**Fig. 5. Comparison of the nucleotide sequence of the rat TrxR2 SECIS element with those of SECIS elements of other selenoproteins (A) and the predicted secondary structure of the rat TrxR2 SECIS element (B).** A. The rat TrxR2 SECIS element was compared with those of rat and human TrxR1 (18); human and murine 15-kDa selenoprotein (15K SELP) (63); rat and human deiodinase (43); rat, human, murine, and bovine glutathione peroxidase (GPX) (43); and rat and human selenoproteins 1 and 2 (SELP1 and SELP2) (43) with the Pileup program (University of Wisconsin Genetics Computer Group). The position of the first base in each sequence from rat, or in human 15K SELP, is indicated in parentheses. B. The stem-loop structure of the rat TrxR2 SECIS element was generated with the Foldrna program (University of Wisconsin Genetics Computer Group).

50% similarity) as well as to putative GR sequences of *Caenorhabditis elegans* (39) and *Drosophila melanogaster* (40) (48 to 50% identity, 57 to 58% similarity). Furthermore, unlike human and mouse GR proteins, the *C. elegans* and *D. melanogaster* enzymes possess long COOH-terminal regions that are similar to those of mammalian TrxR proteins. The COOH termini of *C. elegans* and *D. melanogaster* GR proteins also end with the sequences GCCG and SCCS, respectively, which resemble the GCUG motif at the COOH termini of mammalian TrxR enzymes. As a result of the effort to sequence *C. elegans* chromosome III (41), another putative GR gene (GenBank accession number, U61947) has been identified on the basis of its homology (36% identity and 45% similarity) to mammalian GR enzymes. However, the gene product has not been shown to possess GR activity, and, at the time the gene was characterized, mammalian TrxR genes had not been identified. Comparison of the predicted amino acid sequence of the protein encoded by the chromosome III gene with those of TrxR1 and TrxR2 revealed higher homology (48 to 59% identity and 58 to 69% similarity) to these proteins than to mammalian GR enzymes. Furthermore, in mammalian TrxR enzymes, the codons for Secys (TGA) and Gly (GGT) in the chromosome III gene are followed by the stop codon TAA, indicating that the gene product could contain the sequence GCUG at its COOH terminus. Therefore, we listed the product of the chromosome III gene as a TrxR rather than as a GR in Fig. 4. TrxR2 showed low homology to other members of the flavoprotein disulfide oxidoreductase family, such as human dihydrolipoamide reductase (42) (28% identity) and *Pseudomonas aeruginosa* mercuric reductase (43) (26% identity).

Secys Insertion Sequence Element in the 3'-Untranslated Region of TrxR2 cDNA—The encoding of Secys by TGA in eukaryotic selenoproteins requires the presence of a Secys insertion sequence (SECIS) element such as that located in the 3'-untranslated regions of transcripts that encode thyroid hormone deiodinases, glutathione peroxidases, and several types of selenoprotein P (44, 45). The spacing between the TGA codon and the SECIS element varies greatly. The SECIS element has been defined on the basis of conserved sequence rather than functional features. The conserved sequence includes the invariable AUGA, three consecutive A residues that are separated by 9 to 12 residues from the AUGA motif, and the doublet GA that is separated by a widely variable number of residues from the AAA triplet (Fig. 5A). Although the overall sequence homology among SECIS elements is low, they exhibit conserved stem-loop structures that can be divided into two types (46). Both type I and type II structures contain the conserved sequences AUGA and GA in the 5' and 3' arms, respectively, of the stems. The two types differ in that the unpaired AAA sequence is located in the apical loop in type I structures but forms a bulge in the 5' arm of type II structures. The AAA-containing bulge in type II structures is separated from the apical loop by a predicted stem of 3 to 5 base pairs. SECIS elements with a long sequence between the AAA triplet and GA dinucleotide show a tendency to assume a type II structure (47, 48).

The 3'-untranslated region of TrxR2 cDNA contains a putative SECIS element that conforms to the consensus sequence described above. A computer folding program indicated that the TrxR2 SECIS element forms a type II stem-loop structure that contains a
FIG. 6. Separation of TrxR2 from TrxR1 by acid precipitation (A) and purification of TrxR2 from the acid-precipitated proteins (B-D). A, immunoblot analysis with antisera to TrxR1 (αTrxR1) and to TrxR2 (αTrxR2) of rat liver homogenate (crude extract) as well as of the supernatant and precipitate obtained after acidification of the homogenate to pH 5.0. B-D, purification of TrxR2 by sequential chromatography of the acid-precipitated proteins on columns of DEAE-Sephacel (B), 2′, 5′-ADP-agarose (C), and Phenyl-5PW (D). Column fractions were subjected to immunoblot analysis with antibodies to TrxR2 (insets). See “Experimental Procedures” for further details.
were improved as described under “Experimental Procedures.” The modified approach, which includes three successive column chromatographies after the acid precipitation step, allowed us to obtain TrxR1 and TrxR2 with no cross-contamination and with higher yields, because increased pooling of column fractions was possible. Elution profiles for the three column steps are shown for the purification of TrxR2 (Fig. 6, B-D). The procedure yielded 6.5 mg of TrxR1 and 2.1 mg of TrxR2 with high purity (>95%) from 1 kg of rat liver.

Homogeneity, Size, and Shape of TrxR Enzymes—Like the TrxR enzymes from prokaryotes and yeast, mammalian TrxR1 was shown to be a dimer in its native state. Analysis by non-denaturing PAGE of TrxR1 and TrxR2 purified by the improved procedure yielded a value for the molecular mass of TrxR1 of 115 kDa, consistent with that expected for a dimer. However, the mobility of TrxR2 was substantially less than that of TrxR1. Electrophoresis performed overnight on an 8 to 16% gradient gel yielded an estimated size of 300 kDa for TrxR2 (data not shown). Furthermore, the molecular mass of TrxR2 determined from its mobility was dependent on the time of electrophoresis. Because the isoelectric point of mature TrxR2 (8.0) predicted from its amino acid sequence is substantially higher than that of TrxR1 (5.9), it was possible that the lower mobility of TrxR2 was due to its lower charge density under the conditions of electrophoresis rather than to a difference in oligomerization state.

To verify this supposition, we subjected TrxR1 and TrxR2 to analytical ultracentrifugation. A single, symmetrical sedimentation boundary was observed for both TrxR1 and TrxR2 at pH 7.4 and 20 °C. Time derivative analyses of four late concentration profiles are shown for TrxR1 (Fig. 7A) and TrxR2 (Fig. 7B). The fit of $g(s^*)$ data for TrxR1 and TrxR2 to a single Gaussian curve in each instance demonstrated the homogeneity of the proteins. The $g(s^*)$ function for TrxR2 (Fig. 7B) appeared broader than that for TrxR1 (Fig. 7A) because TrxR2 was sedimented for a longer time and at a lower speed (40,000 rpm, 93 min) than was TrxR1 (48,000 rpm, 77 min) on the basis of the gel electrophoresis data indicating that TrxR2 might be larger than TrxR1.

The sedimentation properties of TrxR1 and TrxR2 are summarized in Table I. The corrected sedimentation coefficients for TrxR1 and TrxR2 were 6.08 and 6.29 S, and the diffusion coefficients ($±4%$) were $4.85 \times 10^{-7}$ and $5.57 \times 10^{-7}$ cm$^2$/s, respectively, as calculated from Gaussian fits of the $g(s^*)$ distributions shown in Fig. 7. These values and the partial specific volumes calculated from the amino acid compositions (29) yielded molecular weight values within 4% of those expected for dimers of TrxR1 and TrxR2. The determined frictional coefficients ($f_r$) were slightly higher than those ($f_R$) calculated for spherical dimer particles, indicating that shape or volume effects reduce sedimentation rates.

Catalytic Activity—The broad substrate specificity of mammalian TrxR proteins has allowed the NADPH-dependent reduction of 5,5'-dithiobis(2-nitrobenzoic acid) to be used as the basis for an assay of TrxR activity (14, 15). In the present study, we have used an assay of greater physiological relevance that is based on the reduction of recombinant mammalian Trx after its oxidation by H$_2$O$_2$. The specific activities of TrxR1 and TrxR2 measured in the presence of saturating concentrations of oxidized Trx and NADPH were 2.2 and 3.3 nmol/min/mg of protein, respectively (Fig. 8A). The COOH-terminal Secys of TrxR1 was recently shown to be essential for catalytic activity (18, 35). To determine whether the Secys residue of TrxR2 was similarly essential, freshly prepared TrxR2 was labeled with BIAM at pH 6.5 as described under “Experimental Procedures” and Fig. 2, with the exception that the BIAM-labeled enzyme

![Graphical representation](image)

**Fig. 7.** Sedimentation velocity analysis at 20.0 °C and time derivative analysis of four late concentration profiles at 280 nm for TrxR1 (A) and TrxR2 (B), with harmonic average sedimentation times of 76.6 and 92.8 min at 48,000 and 40,000 rpm, respectively. The concentrations of TrxR1 and TrxR2 were 0.92 and 1.05 mg/ml, respectively. Unsmoothed data from triple-averaging in 0.003-cm steps (open circles) in each panel show the apparent distribution function, $g(s^*)$, versus the sedimentation coefficient ($s^*$) in Svedberg units (S). The solid line in each panel represents a single Gaussian fit for either the TrxR1 or TrxR2 data set. The observed sedimentation and diffusion coefficients of the solute are given by the $s^*$ values at the maximum and the half-width, respectively, of the $g(s^*)$ curve (28, 29).

**TABLE I**

**Sedimentation properties of TrxR1 and TrxR2**

| Sample | $M_{s,\text{main}}$ (calculated) | $s_{20,w}$ (S ± 0.08) | $M_r$ (±4%) | $f/f_0$ |
|--------|---------------------------------|-----------------------|-------------|---------|
| TrxR1  | 54,491                          | 6.08                  | 111,000     | 1.4     |
| TrxR2  | 53,036                          | 6.29                  | 102,000     | 1.3     |

AAA bulge and a stem of three GC pairs below the apical loop of six nucleotides (Fig. 5B).

**Improved TrxR Purification Procedure**—A rabbit antiserum to TrxR2 was prepared by injection with a peptide (RSGLDPTVTGCGC) that is identical to the COOH-terminal sequence of TrxR2 with the exception that Secys was replaced by Cys. Immunoblot analysis with this antipeptide serum and with rabbit antiserum to TrxR1 indicated that the acidification of rat liver homogenate to pH 5 resulted in the precipitation of TrxR2, whereas TrxR1 remained soluble (Fig. 6A). On the basis of this result, the purification protocols for TrxR1 and TrxR2

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was not subsequently exposed to iodoacetamide. Labeling with BIAM completely blocked the activity of TrxR2 toward oxidized Trx (Fig. 8B). Labeling of TrxR2 as described in Fig. 2 yielded only one major BIAM-labeled peptide, in which Secys, but not the adjacent Cys, was modified. Like other flavoprotein disulfide oxidoreductases, both TrxR1 and TrxR2 contain a redox-active disulfide center comprising the sequence CVNVGC (residues 52 to 57 in mature TrxR2). However, the two cysteine residues in this sequence were not labeled by BIAM in the experiment shown in Fig. 2. These results suggest that the inactivation of TrxR2 by BIAM resulted from modification of the Secys residue, and thus that this residue is essential for the catalytic activity of this protein. Because of the high homology between TrxR2 and GR enzymes from human, C. elegans, and D. melanogaster, we also assayed TrxR2 for GR activity with yeast GR as a control. TrxR2 did not exhibit detectable GR activity (Fig. 8C).

**Selenium Content of TrxR Enzymes**—The selenium content of TrxR1 purified from various sources was previously determined to be 0.6 to 0.93 mol of selenium per subunit (4, 18). TrxR appears to lose selenium under conditions of increased oxidative stress, as indicated by the observation that the selenium content of TrxR1 from HeLa cells decreased by almost half when the oxygen level in the culture chamber was increased (35). We measured the selenium content of freshly purified TrxR1 and TrxR2 by atomic absorption spectrometry and comparison with dilutions of a standard selenium stock solution. Five independent measurements with TrxR enzymes in the concentration range of 13.5 to 40.5 μg/ml yielded selenium contents of 0.75 ± 0.08 and 0.84 ± 0.20 mol of selenium per subunit (means ± S.E.) for TrxR1 and TrxR2, respectively.

**Tissue Distribution and Subcellular Localization of TrxR Isoforms**—Total soluble fractions of sonicates prepared from various rat tissues were subjected to immunoblot analysis with rabbit antibodies specific for TrxR1 or TrxR2 (Fig. 9A). Comparison of the intensities of the immunoreactive proteins in the various tissues with those of purified TrxR proteins allowed us to estimate the amount of each isoform in micrograms of TrxR per milligram of total soluble protein. TrxR1 was abundant in all the tissues examined, varying in amount from 0.6 to 1.6 μg/mg of soluble protein. However, TrxR2 was relatively abundant (0.3 to 0.6 μg/mg of soluble protein) only in liver, kidney, adrenal gland, and heart; in the other tissues, the amount of TrxR2 was below the limit of detection (0.02 μg/mg).

The antibodies to TrxR2 detected two bands in the liver and kidney (Fig. 9A). The lower band corresponded to TrxR2. Longer exposure of the immunoblot also revealed a faint upper band for the other tissues. This upper band might correspond to a protein that shows low cross-reactivity with the antibodies to TrxR2. However, the marked intensity of the upper band in kidney is suggestive of the presence of a third isoform of TrxR that is similar in size to TrxR1 but which possesses a COOH-terminal sequence highly similar to that of TrxR2. Alternatively, the upper band might represent the TrxR2 preprotein.

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**Fig. 8. Measurement of TrxR and GR activities of TrxR2.** A, the Trx-reducing activities of 1 μg of TrxR1 (■) and TrxR2 (▲) were measured by coupling the reduction of oxidized Trx to NADPH oxidation and monitoring the decrease in A340. An assay mixture lacking enzyme served as a control (●). B, the Trx-reducing activities of 1 μg of BIAM-labeled (■) or unlabeled (▲) TrxR2 were measured as in A. An assay mixture lacking TrxR2 served as a control (●). TrxR2 was labeled with BIAM as described under "Experimental Procedures," with the exception that subsequent incubation with iodoacetamide was omitted. C, the GR activities of 2 μg of yeast GR (▼), rat TrxR1 (■), and rat TrxR2 (▲) were measured by coupling the reduction of GSSG to NADPH oxidation and monitoring the decrease in A340. An assay mixture lacking enzyme served as a control (●).
with the NH₂-terminal 36 residues intact, which would suggest that the translocation of the preprotein from the cytosol into mitochondria is slower in kidney than in other tissues.

We next investigated the subcellular localization of TrxR1 and TrxR2 by immunoblot analysis of cytosolic and mitochondrial fractions of rat liver (Fig. 9B). Whereas TrxR1 was detected only in the cytosolic fraction, TrxR2 was present predominantly in the mitochondrial fraction.

**DISCUSSION**

While the present study was in progress, Rigobello et al. (19) described the purification of TrxR from a rat liver mitochondrial fraction. Like previously isolated mammalian TrxR enzymes (15), the purified mitochondrial enzyme exhibited a broad substrate specificity. However, its chromatographic behavior differed from that of the cytosolic enzyme and its size was smaller than that of the cytosolic enzyme. It was not determined whether the purified mitochondrial protein was derived from the cytosolic enzyme or whether it contained a Secys residue.

We have now established a relatively simple procedure for the purification of TrxR1 and TrxR2 without cross-contamination, and we have cloned a cDNA encoding rat TrxR2. Comparison of the amino acid sequence deduced from the cDNA with the experimentally determined sequence of the NH₂-terminal region of purified TrxR2 indicated that TrxR2 is likely synthesized in the cytoplasm as a pre-protein that is converted to the mature form in mitochondria by removal of the 36 NH₂-terminal residues. Like TrxR1, but unlike TrxR proteins from prokaryotes and yeast, TrxR2 contains an essential Secys residue in the COOH-terminal region. As in other selenoproteins, the Secys residue of TrxR2 appears to be encoded by a UGA codon under the influence of a stem-loop structure formed by a SECIS element located in the 3′-untranslated sequence of TrxR2 mRNA.

Mammalian cells express two distinct forms of superoxide dismutase, cytosolic CuZn-superoxide dismutase and mitochondrial Mn-superoxide dismutase. We have previously shown that mammalian cells express a family of peroxidases, termed the peroxidin family, that reduce H₂O₂ and lipid peroxides with the use of electrons donated by Trx (26, 49). On reaction with hydroperoxides, the redox-sensitive Cys residue of Prx is oxidized to Cys-SOH, which then reacts with a neighboring Cys-SH of the other subunit to form an intermolecular disulfide. This disulfide is specifically reduced by Trx, but not by glutathione or glutaredoxin (5, 26). Whereas Prx I, II, and IV isoforms are cytosolic proteins, Prx III is synthesized in the cytosol and then transferred to mitochondria, where its 62 or 63 NH₂-terminal residues are cleaved during maturation (26, 50, 51). Like TrxR2, Prx III is most abundant in adrenal gland, heart, liver, and kidney.² Recently, a cDNA that encodes a second isoform of Trx (Trx2) with a 60-residue mitochondrial targeting sequence was cloned and its specific expression in mitochondria confirmed (25).

Most of the reactive oxygen species generated in unstimulated mammalian cells are generated as a result of the univalent reduction of molecular oxygen to the superoxide anion (O₂⁻) by electrons that leak from the mitochondrial respiratory chain; the O₂⁻ then undergoes spontaneous or enzyme-mediated dismutation to H₂O₂. Adrenal cortical mitochondria contain several cytochrome P-450 enzymes that participate in the hydroxylation of cholesterol during the synthesis of adrenal steroid hormones (52). The cytochrome P-450 electron carrier system also leaks electrons and generates O₂⁻ and H₂O₂. Thus, our discovery of TrxR2, which presumably functions together with Mn-superoxide oxidase, PrxIII, and Trx2, highlights a mitochondria-specific defense system against O₂⁻ and H₂O₂.

Increased oxidative stress in mitochondria results in collapse of the mitochondrial membrane potential, consequent impairment of oxidative phosphorylation of ADP, and, ultimately, cell death (53, 54). Oxidative stress in mitochondria also promotes the calcium-dependent, nonspecific permeabilization of the inner membrane as a result of the oxidation and cross-linking of thiol groups in membrane proteins (55, 56). Such increased nonspecific permeabilization has been suggested to lead to the

² S. W. Kang and S. G. Rhee, unpublished observation.
release of mitochondrial constituents, including cytochrome $c$, into the cytosol, which in turn induces cell death by apoptosis (57–61). Thus, the line of defense provided by PrxIII, Trx2, and TrxR2 against H$_2$O$_2$ likely plays a critical role in cell survival.

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REFERENCES

1. Holmgren, A. (1985) Annu. Rev. Biochem. 54, 257–271
2. Nakamura, H., Nakamura, K., and Yodoi, J. (1997) Annu. Rev. Immunol. 15, 369–396
3. Thelander, L., and Reichard, P. (1979) Annu. Rev. Biochem. 48, 133–158
4. Tamura, T., Gladyshev, V., Liu, S.-Y., and Stadtman, T. C. (1996) BioFactors 7, 17045–17054
5. Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) J. Biol. Chem. 269, 27670–27678
6. Lee, S.-R., Kwon, K.-S., Kim, S.-R., and Rhee, S. G. (1998) J. Biol. Chem. 273, 15366–15372
7. Abate, C., Patel, L., Rauscher, F. J., and Curran, T. (1990) Science 249, 1157–1161
8. Hayashi, T., Ueno, Y., and Okamoto, T. (1993) J. Biol. Chem. 268, 11380–11388
9. Schenk, H., Klein, M., Erdbrügger, W., Drioge, W., and Schulze-Osthoff, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1672–1676
10. Hori, K., Matsui, M., Iwata, S., Nishiya, A., Mori, K., and Yodoi, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3633–3638
11. Machado, A. K., Morgan, B. A., and Merrill, G. F. (1997) J. Biol. Chem. 272, 17045–17054
12. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) EMBO J. 17, 2596–2606
13. Wakasugi, N., Tagaya, Y., Wakasugi, A., Mitsu, M., Maeda, Y., Yodoi, J., and Tursz, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8282–8286
14. Tamura, K., and Stadtman, T. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1096–1011
15. Luthman, H., and Holmgren, A. (1982) Biochemistry 21, 6628–6633
16. Russell, M., and Molder, P. (1988) J. Biol. Chem. 263, 9015–9019
17. Chae, H. Z., Robinson, K., Poole, L. B., Church, G., Storz, G., and Rhee, S. G. (1994) J. Biol. Chem. 269, 10717–10721
18. Zhong, L., Arner, E. S. J., Ljung, J., Askland, F., and Holmgren, A. (1998) J. Biol. Chem. 273, 8581–8589
19. Bigi, M. P., Callegaro, M. T., Barzon, E., Benetti, M., and Bindoli, A. (1996) Free Radical Biol. & Med. 24, 370–376
20. Gasdaaska, P. Y., Gasdaska, J. R., Cochran, S., and Powsis, G. (1995) FEBS Lett. 373, 269–272
21. Gladyshev, V. N., and Stadtman, T. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6146–6151
22. Stadtman, T. C. (1996) Annu. Rev. Biochem. 65, 83–100
23. Liu, S.-Y., and Stadtman, T. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6138–6141
24. Arscott, L. D., Groner, S., Schirmer, R. H., Becker, K., and Williams, C. C., Jr. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3621–3626
25. Spyrou, G., Enmark, E., Miranda-Vizuete, A., and Gustafsson, J.-Å. (1997) J. Biol. Chem. 272, 2936–2941
26. Kang, S. W., Chae, H. Z., Lee, M. S., Kim, K., Baines, I. C., and Rhee, S. G. (1996) J. Biol. Chem. 271, 6297–6302
27. Apfel, A., Fischer, S., Goldberg, G., Goodley, P. C., and Kuhlmann, F. E. (1995) J. Chromatogr. A 712, 177–190
28. Shapirio, B. M., and Ginzburg, A. (1968) Biochemistry 7, 2153–2167
29. Zamyatnin, A. A. (1984) Rev. Recent Progr. HIV 13, 145–165
30. Stafford, W. F., III (1996) Biophys. J. 70, A231
31. Stafford, W. F., III (1997) Curr. Opin. Biotechnol. 8, 14–24
32. Daher, R., and van Lente, F. (1994) Clin. Chem. 40, 62–70
33. Hakkola, E. H., Autio, H. H., Sormunen, R. T., Hassanen, I. E., and Hiltunen, J. K. (1989) J. Histochem. Cytochem. 37, 1863–1867
34. Svensson, L. T., Alexson, S. E. H., and Hiltunen, J. K. (1995) J. Biol. Chem. 270, 12177–12183
35. Gorlatov, S. N., and Stadtman, T. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8520–8525
36. Netzer, W. (1997) Annu. Rev. Biochem. 66, 863–917
37. Hendrick, J. P., Hodges, P. E., and Rosenberg, L. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4056–4060
38. Tutic, M., Lu, X., Schrimer, R. H., and Wer, D. (1999) Eur. J. Biochem. 155, 528–532