HUMAN LENS THIOLTRANSFERASE: CLONING, PURIFICATION, AND FUNCTION

Fengyu Qiao,1,2,5 Kuiyi Xing,1,2 Aimin Liu,1 Nancy Eblers,1 Nalini Raghavachari,1,4 and Marjorie F. Lou1,5,6

PURPOSE. To clone the human lens thioltransferase (TTase) gene and to purify, characterize and study the possible function of the recombinant human lens thioltransferase (RHLT).

METHODS. The human lens TTase gene was cloned by using RT-PCR and verified by sequence and RNase protection assay. TTase overexpressed in Escherichia coli was isolated and purified to homogeneity by column chromatography and identified by Western blot analysis. The activity was assayed with a synthetic substrate hydroxyethyl disulfide. Its function in de-thiolating and reactivating other key metabolic enzymes was studied by using pure glutathione S-transferase (GST) and glutathione peroxidase (GPx) from commercial source and also with the cell extract of rabbit lens epithelial cells preexposed to H2O2.

RESULTS. The cloned human TTase gene showed identical sequence to the TTase gene from other human tissues. The RNase protection assay displayed a single transcript from the total RNA of human lens epithelial cells. The purified RHLT had a molecular weight of 11.8 kDa and reacted positively with anti-pig liver TTase. It displayed similar structural, functional, and kinetic characteristics to those of TTases from other sources. It was shown that RHLT effectively regenerated the activities of GST and GPx, after each was inactivated by S-thiolation with cystine in vitro. Furthermore, RHLT was able to restore the activity of the oxidatively inactivated glyceraldehyde-3-phosphate dehydrogenase (G-3PD) in H2O2-exposed rabbit lens epithelial cells.

CONCLUSIONS. The human lens TTase gene has been cloned for the first time. Its gene product showed the characteristics which support our speculation that TTase may play a major role in maintaining the homeostasis of lens protein thiols thus protecting against oxidative stress. (Invest Ophthalmol Vis Sci. 2001;42:743–751)

TTase (EC 1.8.4.3) is a cytosolic enzyme that belongs to the family of thiol-disulfide oxidoreductases (TDORs).1,2 This low-molecular-weight (11.8 kDa) enzyme has been purified and characterized in various tissues3–9 as well as cloned and overexpressed from several species.10–16 TTase is classified generally as TDOR enzyme because it has thiol groups in the active center and participates in catalyzing dithiol–disulfide reductions of low-molecular-weight disulfides and protein–thiol mixed disulfides, assisted by glutathione.1,2 TTase thus may act as a cellular repair enzyme because much of the oxidative damage is attributed to oxidative stress–induced protein S-thiolation9 suggest that there may be a repair system in the lens to abate this oxidative damage. TTase has been speculated to participate in such functional modification of sulfhydryl group of proteins by dithiol and oxidized state (intramolecular disulfide), exerts a reducing power for substrates containing S–S bond and exchanges disulfide to its corresponding thiols. Therefore, it is believed that TTase may play an important role in the maintenance of sulfhydryl homeostasis and regulation of enzyme activities.1,2,18

Although TTase has been studied extensively in many tissues, only recently has it been detected in the lens9 and other ocular tissues.20 Eye lens is a protein-rich tissue with high sulfhydryl (SH) groups that are extremely vulnerable to oxidative damage. The demonstration of TTase in the lens has great implication, because oxidation is speculated to be one of the main factors for age-related cataracts in humans.21–23 The formation of mixed disulfides between nonprotein thiols and protein SH groups decreases the availability of SH groups for protein–protein disulfide (PSSP) crosslinks. These changes in the lens may lead to protein insolubility, transparency loss, and cataract formation.27 Hence, maintenance of the redox status of the lens organ is vital for preserving its transparency and physiological function. To prevent lens proteins from aggregation, the thiol groups of the proteins must be protected from oxidation, and the S-thiolated proteins must be restored to their normal, reduced state by effectively cleaving the protein–thiol mixed disulfides.

Cui and Lou25 and Padaonkar et al.28 reported that if the oxidant was removed from the environment of an oxidant preexposed lens, the elevated protein–thiol mixed disulfides in the lens could spontaneously diminish to a low level, as found in an undisturbed normal lens. These observations along with the fact that lens epithelial cells could also recover from oxidative stress–induced protein S-thiolation27 suggest that there may be a repair system in the lens to abate this oxidative damage. TTase has been speculated to participate in such repair because much of the oxidative damage is attributed to functional modification of sulfhydryl group of proteins by disulfide bond formation. TTase thus may act as a cellular repair enzyme by catalyzing the reduction of disulfide bonds in these S-thiolated proteins or enzymes.

To prove that lens TTase has a repair function for S-thiolated proteins or enzymes, it is essential to use purified TTase for various enzyme kinetics and functional studies. Because of the relatively low ratio of TTase protein to total proteins in the lens, it was difficult to obtain lens TTase in high purity.29 For

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this reason, we chose an alternative approach by using recombinant human lens TTase (RHLT) for the study. This article describes the cloning and sequence analysis of the human lens TTase gene, its overexpression in Escherichia coli, and the purification and characterization of RHLT. The function of RHLT in regenerating activities of several key metabolic enzymes that are inactivated by S-thiolation during oxidative stress is also described.

**Materials and Methods**

NADPH, NADH, ATP, GSH, glutathione reductase (GR), lysozyme, thioredoxin (TRx), thioredoxin reductase (TR), Glycerate 3-phosphate (CHA salt), 3-phosphoglyceric phosphokinase, glutathione S-transferase (GST), glutathione peroxidase (GPx), iodoacetamide (IAA), H$_2$O$_2$, phenylmethylsulfonyl fluoride (PMSE), ethylenediaminetetraacetic acid (EDTA), diethiothreitol (DTT), rabbit serum, fetal bovine serum (FBS), cell culture medium (MEM), gentamicin, acrylamide, and formaldelyde were all from Sigma Chemical Co. (St. Louis, MO). Hydroxyethyl disulfide (HEDS) was purchased from Aldrich-Chemie (Milwaukee, WI). S-sulfoxycysteine was a gift from Alcon Laboratories (Fort Worth, TX) and was synthesized according to the procedure of Segel and Johnson. Sephadex-G-75 and Q-Sepharose FF was obtained from Pharmacia (Upp sala, Sweden). Bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL).

[25P]UTP was from ICN Biomedicals, Inc. (Costa Mesa, CA). Agarose, Taq DNA polymerase, EcoRI, NdeI, and isopropyl-b-D-thiogalactoside (IPTG) were all purchased from Gibco-BRL (Grand Island, NY). Eukaryotic TA cloning kit (unidirectional) and cDNA cycle kit were from Invitrogen (Carlsbad, CA). Riboprobe in vitro transcription systems and β-actin primers were from Promega Corp. (Madison, WI). QIAfilter plasmid midi kit, QIAquick nucleotide removal kit, and QIAquick gel extraction kit were obtained from Qiagen, Inc. (Valencia, CA). BugBuster protein extraction reagent and pET System were from Novagen, Inc. (Madison, WI). RNase protection assay kit was from Torrey Pines Biolabs, Inc. (San Diego, CA). Immunoblot assay kit, nitrocellulose membrane, electrophoresis protein standards, isoelectric focusing (IEF) standards, and ampholytes were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals and reagents were of analytical grade.

Human lens epithelial cell line, HLE B3, was a gift from Usha Andley of Oakland University (Rochester, MI). Rabbit lens epithelial cell line N/N1003A was a gift from John Reddan of Oakland University (Rochester, MI). Human lens epithelial cells (B3) were grown in MEM medium with 20% FBS containing gentamicin in a humidified CO$_2$ incubator. Total RNA was extracted form fresh cells with an RNA isolation kit and measured for absorbance at 260 and 280 nm. The purity of RNA was further analyzed on formaldehyde agarose gel.

**Cloning of Human Lens TTase**

Human lens TTase cDNA was isolated by RT-PCR technique. Primers were designed on the basis of the human placenta TTase gene sequence (GenBank accession number x76648) and obtained from Integrated DNA Technologies, Inc. (Coralville, IA): forward primer (sense): 5’-GGATGGGCTCAAGAGTTTGTG-3’, reverse primer (antisense): 5’-TCTCTATGACATGCCTGTTAACTTATG-3’. The first cDNA strand was generated using cDNA cycle kit (Invitrogen), following the instruction manual. An aliquot of total RNA from above (11.5 µl or 5 µg) was mixed with 1 µl of random primers. The mixture was heated in a 65°C incubator for 10 minutes and chilled on ice, followed by addition of the following reagents: 1 µl of RNase inhibitor, 4 µl of 5X RT buffer, 1 µl of 100 mM dNTPs, 1 µl of 80 mM sodium pyrophosphate, and 0.5 µl of AMV reverse transcriptase. The reaction was conducted in a 42°C water bath for 60 minutes. An aliquot (2 µl) of the reverse transcriptase reaction mixture was used as a template for PCR. The amplification was performed in a total volume of 50 µl containing 5 µl of 10X PCR buffer, 1.5 µl of 50 µM MgCl$_2$, 1 µl of 100 mM dNTPs, 1 µM of each primer, and 1 unit of Taq DNA polymerase (Gibco-BRL). The reaction was performed for 30 cycles, with 45 seconds at 94°C, 1 minute at 55°C, and 2 minutes at 72°C followed by an additional 5 minutes at 72°C and was maintained at 4°C. The RT-PCR products were analyzed on 1% agarose gel. β-Actin amplified with primers from Promega was used as internal control.

PCR products were purified and cloned in pCR3.1-uni vector and the plasmid DNA transformation was prepared and sequenced at the DNA Sequencing Facility (Iowa State University). The sequence was compared with published TTase sequences.

**RNase Protection Assay**

To synthesize the probe for the RNase protection assay, recombinant plasmids containing the human lens TTase cDNA in pCR3.1-uni vector in both directions—pCR3.1-uni/HLT-F3B and pCR3.1-uni/HLT-Rev—were each purified by using QIAfilter midi-kit. Linear plasmids were obtained by digestion with EcoRI followed by recovery from agarose gel. Both [32P]UTP–labeled antisense RNA and sense RNA probes were synthesized using Riboprobe in vitro transcription kit, and the labeled probes were purified by using the QIAquick nucleotide removal kit. The specific activity of the labeled probes was determined using the Pharmacia Wallac 1410 Liquid Scintillation Counter. The probe with 1.0 × 10$^8$ dpm was used for each assay.

The RNase protection assay was performed using a kit from Torrey Pines Biolabs, Inc., following the instruction manual. In brief, 12 µg of the total RNA from HLE B3 cells was hybridized with either antisense RNA probe or sense RNA probe for 25 minutes at 90°C. The hybridization solutions were incubated with RNase A/T1 at room temperature for 30 minutes. The protected RNA was precipitated and separated on 6% sequencing gel by electrophoresis, and the gel was then exposed to x-ray film for 4 days.

**Overexpression of RHLT in E. coli**

pET expression system from Novagen (Madison, WI) was used to express the human lens TTase in E. coli. To obtain the protein product without the extra residues in the N-terminal end, PCR technique was used to introduce the Ndel site into the 5’ end of the human lens TTase sequence (GenBank accession number x76648) and its original reverse primer, using sequenced insert in pCR3.1-uni vector as the template. The above PCR product was again cloned into pCR3.1-uni vector, and its sequence was confirmed by sequencing. The positive plasmid was purified, and its insert was recloned into pET23a(+) between Ndel and EcoRI sites. After sequencing, the positive clone was used for high-level expression in BL21 (DE3) by IPTG induction. By following the instruction manual from the pET System (kit) provided by Novagen, one single colony of E. coli with the inserted plasmid containing human lens TTase cDNA was inoculated in 40 ml LB medium with 100 µg/ml ampicillin and grown overnight. Ten milliliters of the overnight grown cells was placed in 1 liter of LB media containing 100 µg/ml ampicillin and grown for 6 hours until the cell density reached an absorbance (600 nm) level of 0.4 to 0.6. IPTG (0.5 mM) was added, and the culture was grown for 0, 0.5, 1, 2, 3, 4, 6, and 8 hours. The cells were collected by centrifugation at 13,000 rpm for 30 seconds, and the pellet was resuspended in 500 µl of Bugbuster reagent (Novagen) and incubated for 10 minutes before centrifuging again at 13,000 rpm for 8 minutes. The supernatants were saved for protein measurement, TTase activity assay, and other experiments. The BL21 (DE3) cells without plasmid served as controls.

**Purification of RHLT**

An FPLC system (LKB/Pharmacia, Piscataway, NJ) equipped with a UV detector and a fraction collector was used for the following chromato-
graphic procedures. All operations were done at 4°C, unless otherwise mentioned. An aliquot of the above cell extract (3.5 ml) was loaded onto a Sephadex G-75 column (2.5 × 120 cm), pre-equilibrated with 10 mM, pH 7.4, potassium phosphate buffer, and eluted with the same buffer. Fractions with TTase activity were pooled and concentrated with Amicon YM 3 (Amicon Inc., Beverly, MA). This partially purified enzyme was then loaded onto a QA-Sepharose FF column (1.6 × 17 cm) and eluted with 10 mM, pH 5.8, potassium phosphate buffer. The fractions with TTase activity were again pooled, concentrated with Centricon 3 (Amicon Inc.), and then stored at −80°C for additional studies.

**Assay for TTase**

The standard assay for TTase followed the method of Mieyal et al., which was modified by Raghavachari and Lou. Briefly, the reaction mixture contained 0.2 mM NADPH, 0.5 mM GSH, 0.1 M potassium phosphate buffer (pH 7.4), 0.4 units of GR, and an aliquot of either crude, partially purified, or purified RHLT in a total volume of 1 ml. The reaction was carried out at 30°C and initiated after a 5-minute preincubation with 2 mM HEDS. The decrease in absorbance of NADPH at 340 nm was monitored for 5 minutes, using spectrophotometer (model DU 640; Beckman, Fullerton, CA). The slope of the linear portion of the time course for A340 nm absorption loss in control (TTase free) was subtracted from the slope of the samples (containing TTase) and was used to determine TTase activity. One unit of TTase activity is defined as 1 μmol of NADPH oxidized/min. (i.e., A340 nm for NADPH = 6.2 mM−1 cm−1, which is the molar extinction coefficient of NADPH) under these standard assay conditions.

**Protein Assays**

Protein concentration was determined with BCA method following the manufacturer’s protocol (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard.

**SDS-PAGE, Immunoblotting, and N-terminal Amino Acid Sequencing**

Electrophoresis of partially purified and purified RHLT on vertical polyacrylamide slab gels was performed in the presence of SDS by the method of Laemmli. The resolving gel was homogeneous at 12% of acrylamide and the stacking gel was 4.3% of acrylamide. The electrophoresis was performed at 120 V for 80 minutes. Broad molecular weight standard from Bio-Rad (Hercules, CA) was used for this assay. The protein bands were stained and visualized by Coomassie Brilliant Blue R-250.

Western blot analysis was performed by the method of Towbin et al. The purified RHLT was first subjected to electrophoresis with 12% SDS-PAGE and then electroblotted at 100 V/h onto a nitrocellulose sheet. Anti-pig liver TTase (1:1000 dilution) was used as the first antibody. The resolved gel was homogenous at 12% of acrylamide and the stacking gel was 4.3% of acrylamide. The electrophoresis was performed at 120 V for 80 minutes. Broad molecular weight standard from Bio-Rad (Hercules, CA) was used for this assay. The protein bands were stained and visualized by Coomassie Brilliant Blue R-250.

**Kinetic Studies**

Studies for substrate concentration dependence were carried out as in the standard assay for HEDS, L-sulfinocysteine, and cystine. Substrate concentrations in the range of 0.02 to 1.65 mM was assayed with 0.02 units of RHLT. The apparent and values were calculated based on the Lineweaver-Burk plot.

The effect of GSH on TTase assay was carried out by using GSH concentrations between 0.05 to 0.75 mM in the absence and presence of RHLT.

**Effect of IAA on RHLT Activity**

Aliquot of reduced 0.02 units of RHLT (pretreated with 0.5 mM DTT) was incubated with 30 μM IAA in 50 μl of 0.1 M potassium phosphate buffer, pH 7.4, for various time intervals (between 0 and 30 minutes) before the mixture was used for TTase assay. For protection studies, the same amount of reduced RHLT as above was preincubated with either 2.5 mM L-sulfinocysteine or 0.5 mM GSH for 10 minutes at room temperature and then incubated for various time intervals with 30 μM IAA in the same buffer as above. The unreacted TTase was immediately assayed for activity.

**Effect of H$_2$O$_2$ on RHLT Activity**

Purified RHLT was reduced with 4 mM DTT at room temperature for 30 minutes and filtered through PD-10 column (Pharmacia Biotech AB, Uppsala, Sweden) to remove DTT. An aliquot of reduced RHLT (0.02 units) in 10 mM, pH 7.4, potassium phosphate buffer was preincubated with various concentrations of H$_2$O$_2$ (0.05–1.5 mM) in a total volume of 6 μl for 5 minutes at 25°C. Ten microliters of catalase (2 μg) was added at the end of the reaction for 5 minutes to detoxify the remaining H$_2$O$_2$ at the same temperature. The final RHLT activity was determined as described above for TTase standard assay. To examine if H$_2$O$_2$-treated RHLT could be reactivated by reducing agents, RHLT (0.2 units) was incubated with 0.33 mM H$_2$O$_2$ in 120 μl of 10 mM potassium phosphate buffer, pH 7.4, for 5 minutes at 25°C. The oxidation was stopped by adding 2 μg catalase. The sample was then divided into six portions, and each portion was mixed with 0.1 mM phosphate buffer, 5 mM DTT, 0.5 mM GSH, 5 mM GSH, thioredoxin–thioredoxin reductase plus NAPDH (designated as theTrx–Tr system) and Trx alone, respectively, and incubated at 25°C for 20 minutes. The final volume for each of the regenerating reaction mixture was 40 μl. Ten microliters of each of the sample was used for TTase assay. The concentrations of NADPH, Trx, and TR in the Trx–Tr system were 0.25 mM, 10 μM, and 0.8 μM, respectively. Trx–Tr system without H$_2$O$_2$-inactivated RHLT was used as a control.

**Studies of RHLT Function**

The function of RHLT was studied by using sulphydryl-sensitive enzymes as models. The enzymes studied below were chosen because they are key metabolic or oxidative defense enzymes in the lens.

**Regeneration of In Vitro Cysteine-Thiolated GST by RHLT**

GST activity was determined based on the method described by Habig et al. To ensure the enzyme was totally reduced, GST (150 mU) was treated with 1 mM DTT at room temperature for 30 minutes in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1 mM EDTA. DTT was then removed with a PD-10 column, equilibrated, and washed with the same buffer. The reduced GST (15 mU) was then S-thiolated with cysteine (PSCC formation) by incubating with 1 mM cystine in 50 μl of the above buffer for 20 minutes at room temperature, and the excess cystine was removed by PD-10 column. For dethiolating the modified GST, 1 mM GSH, 0.5 mM NAPDH, and 0.6 units of GR with or without 0.1 units of the purified RHLT were added into the above S-thiolated GST mixture (0.2 ml). The final solution was then incubated at 30°C for 5 to 30 minutes and used for GST assay. In another experiment, the modified GST was dethiolated with 1 mM, 2.5 mM, and 5.0 mM GSH, with and without the presence of RHLT at 30°C for 20 minutes.

**Regeneration of In Vitro Cysteine-Thiolated GPx by RHLT**

Procedures for cystine inactivation and subsequent RHLT reactivation of GPx were the same as for GST above, except that S-thiolation of GPx was achieved by using 10 mM potassium phosphate buffer, pH 7.4, and the inactivation reaction was conducted for 30
minutes. In another experiment, the dethiolation was done by using physiological concentrations of GSH (1, 2.5, and 5.0 mM), with and without the presence of RHLT. The GPx activity was determined following the method by Spector et al.57

Regeneration of Oxidatively Damaged G-3PD in Rabbit Lens Epithelial Cells by RHLT. Rabbit lens epithelial cells from line N/N1003A was used for this experiment following previously described procedures.57 In brief, confluent cells (0.8 million) grown in MEM plus 20% rabbit serum were incubated overnight in MEM with 1% rabbit serum and then in serum-free MEM for 30 minutes before a bolus of 0.5 mM H2O2 was added. After 15 minutes, the cells were harvested and then used for G-3PD assay. Aliquots of the cell extract were filtered with a PD-10 column to remove small-molecular-weight components and used for G-3PD assay. The dethiolation of inactivated G-3PD was carried out with 1 mM GSH with and without RHLT. In another experiment, GSH and RHLT regeneration studies, following the procedure described above. The dethiolation of inactivated G-3PD was carried out with 1 mM GSH with and without RHLT. In another experiment, GSH at 1.0 and 5.0 mM was used in the presence and absence of RHLT to clarify the dethiolating function of RHLT. G-3PD activity was analyzed following the method described by Bergmeyer et al.58

RESULTS

Cloning and Sequence of Human Lens TTase

Amplification of human lens TTase from total RNA by RT-PCR resulted in a single 341-bp fragment as shown in Figure 1A. This fragment was purified and cloned into pCR3.1-uni vector. The recombinant clone was confirmed for the presence and orientation of the insert by restriction enzyme analysis and by DNA sequencing. The nucleotide and the deduced amino acid sequence of the coding region of the lens TTase is shown in Figure 1B, in which the 341-bp fragment shows an open reading frame of 321 bp, beginning with an ATG start codon and ending with a TAA stop codon. The open reading frame encodes a polypeptide of 106 amino acids starting with methionine, and the predicted molecular weight of the encoded polypeptide is 11.8 kDa. The sequence of lens TTase gene was deposited in GenBank with an accession number AF-162769.

On comparison with the known TTase from other human tissues using GCg program, it was shown that the amino acid sequence of lens TTase was 100% identical with those of placenta TTase (GenBank accession number x76648) and of neutrophil TTase (GenBank accession number s82191). N-terminal amino acid sequencing of the purified enzyme confirmed the above finding (data not shown). Even though there was one nucleotide difference (C versus T), it did not affect the translation. The lens TTase similar to placenta and neutrophil TTases have two nucleotides that are different from the brain TTase sequence (GenBank accession number d21238), with Leu replacing Val at position 96. The human lens TTase has identical amino acid residues in three highly conserved regions (see Fig. 1B), including the active site of the enzyme (CPYC), the sequence of the active site of the enzyme; TVP, the sequence of the hydrophobic region; IGGCS, and the residues in the hydrophobic region (TVP).

RNase Protection Assay
To verify if the TTase cDNA fragment obtained from RT-PCR can indeed express TTase in human lens epithelial cells, a RNase protection assay was performed. Both the [α-32P]UTP-labeled antisense and sense TTase RNA were obtained by using in vitro transcription reaction, with plasmids containing the human lens TTase fragment in the opposite direction, as a template. As shown in Figure 2, after removal of the excess radioisotope, both probes gave one band of 389 bp (based on sequence). The total RNA from lens epithelial cells, placenta TTase, with TVP, the sequence of the hydrophobic region; IGGCS, and the residues of the hydrophobic region (TVP).

High Level Expression of Human Lens TTase in BL21(DE3) Cells
The pET23a (+) system from Novagen was used for high-level expression of human lens TTase in BL21(DE3) cells. To obtain protein product without the N-tag, the NdeI site was introduced into the 5′ region of the TTase fragment by PCR. The PCR product was cloned into pCR3.1-uni vector first and the recombinant plasmid identified as pCR3.1-1-HTT was sequenced and verified for the added NdeI site before the start codon ATG.

FIGURE 1. Human lens TTase gene. (A) RT-PCR amplification of human lens TTase gene. Total RNA of human lens epithelial cells (BS) was used as a template for RT-PCR with the primers designed from the sequence of the human placenta TTase gene. β-Actin was used as a control. Lane 1: 100-bp DNA ladder; lane 2: β-actin fragment; lane 3: TTase fragment. (B) Nucleotide and deduced amino acid sequence of recombinant human lens TTase. The nucleotides are numbered from 5′ to 3′. The sequence of the PCR primers used are underlined. The initiation methionine and the termination signal are in bold marked with asterisks. CPYC, the sequence of the active site of the enzyme; TVP, the sequence of the hydrophobic region; IGGCSD, and the residues of the hydrophobic region (TVP).
pCR3.1-HLTT was subcloned into pET23a (tor. Positive clones named pET23a (tor) were treated with 10 mM HEDS, the oxidized form became more stable than that of bovine lens TTase. Catalytic activity of RHLT expressed in E. coli cell extract was 29.45 units/mg protein, which was nearly 30,000-fold higher than that of bovine lens TTase. RHLT was well separated from other proteins by using a Sephadex G-75 gel filtration column. This partially purified RHLT was further purified by QA-Sepharose FF column chromatography in which TTase activity was found in the flow-through, whereas most of the other proteins were still bound onto the resin. SDS-PAGE analysis showed a single protein band at molecular weight of 11.8 kDa (Fig. 3A), which reacted positively with anti-pig liver TTase (Fig. 3B). The enzyme was purified up to threefold with 16% yield and a specific activity of 91 units/mg.

**Purification of RHLT**

RHLT-expressed in E. coli BL21(DE3) cells transformed with pET23a(+)-HLTT were confirmed by restriction enzyme analysis, sequencing, and analyzing its gene product. RHLT was partially inactivated when treated with H2O2 in the reaction mixture did not effectively reduce the disulfide substrate HEDS, even at a concentration of 0.25 mM, similar to the observation of TTase of human red blood cells (RBCs). The catalytic efficiency (V_max/K_m) of RHLT was different for the three substrates. RHLT displayed the highest efficiency for cystine, followed by HEDS and S-sulfocysteine. These results are summarized in Table 1.

**Sensitivity of RHLT to IAA and H2O2**

The redox status at the active center of RHLT was sensitive to IAA alkylation. More than 80% inhibition was observed within 15 minutes of IAA treatment, strongly suggesting that the SH group(s) was in the active site. The enzyme was protected from IAA inactivation by preincubating with S-sulfocysteine but not with GSH (data not shown).

**Properties of RHLT**

Both the purified RHLT and the enzyme treated with 10 mM DTT showed a same pl value of 7.2. When the enzyme was treated with 10 mM HEDS, the oxidized form became more basic, and the pl increased to 8.2. This property of two pl's for the two redox status was similar to those reported for both native and recombinant pig liver TTase.15-39 Catalytic activity of RHLT showed a temperature dependence in the range of 30 to 40°C. Therefore, 30°C was used throughout the standard assays. The activity of RHLT for pH dependence was limited to a narrow range, with an optimal pH at 7.5.

The purified RHLT could be inhibited (data not shown) by substrates (HEDS, S-sulfocysteine or l-cystine) with concentrations higher than 0.25 mM, similar to the observation of TTase from other tissues.8,39,40 The K_m values for HEDS, S-sulfocysteine, and l-cystine were calculated to be 0.60, 0.71, and 0.25 mM, respectively, and were similar to those obtained from TTase of human red blood cells (RBCs).40

**Kinetic Summary of Recombinant Human Lens Thioltransferase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m (mM)</th>
<th>V_max (K_m) (min^-1)</th>
<th>V_max/K_m (mM^-1 min^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEDS</td>
<td>0.60</td>
<td>608</td>
<td>1013</td>
</tr>
<tr>
<td>S-sulfocysteine</td>
<td>0.71</td>
<td>310</td>
<td>437</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.25</td>
<td>1010</td>
<td>4040</td>
</tr>
</tbody>
</table>

An increasing concentration of GSH (0.05-0.75 mM) without RHLT in the reaction mixture did not effectively reduce the disulfide substrate HEDS, even at a concentrations approaching the physiological level (0.75 mM). In contrast, increasing concentration of GSH, in the presence of RHLT (fixed amount), exponentially increased the reduction of HEDS. For instance, the rate of substrate reduction by 0.75 mM GSH was only one tenth of the rate when RHLT was present (Fig. 4).
served if the enzyme was first suspended in a larger volume of buffer solution before H$_2$O$_2$ treatment. The activity could be almost fully regenerated by treating with high concentrations of reducing agents, DTT or GSH. But it can also be restored effectively by the TRx–TR system (Table 2). For proper control, the TRx–TR system was tested for dethiolase activity with the same substrate but found to be negative. The H$_2$O$_2$-treated RHLT showed no apparent protein change on SDS-PAGE (data not shown), but IEF showed the same pi value as the oxidized enzyme when it was treated with HEDS, indicating that the H$_2$O$_2$-treated RHLT likely had become more basic by forming intramolecular disulfides.

**Dethiolating Function of RHLT**

**Regeneration of Cystine-Inactivated GST by RHLT.** GST was easily inactivated by S-cysteinylation at its active site to form GST-S-S-cysteine. As shown in Figure 5A, S-thiolation caused GST to lose more than 80% of its activity. However, the activity was regenerated to 90% within 20 minutes after RHLT was added to the reaction mixture, whereas only 50% activity was regenerated when GSH alone (1 mM) was used.

In separate experiments, cystine-treated GST, which lost 95% to 97% of its original activity, was incubated with a physiological concentration of GSH for 20 minutes in the reaction mixture without and with the presence of RHLT. GST activity was restored from 5% to 18%, 30%, and 48% at 1, 2.5, and 5.0 mM of GSH, respectively, whereas in the presence of RHLT, GST was correspondingly reactivated from 3% to 41%, 60%, and 78% of the original activity (data not shown).

**Regeneration of GPx Activity by RHLT.** A similar experiment was conducted for GPx (Fig. 5B), in which S-thiolation inactivated GPx to 50% of its original level after incubating with 1 mM cystine for 30 minutes. Addition of RHLT into the reaction mixture restored GPx to 90% of its initial activity within 30 minutes. In contrast, only approximately 70% of the activity could be regenerated by simple GSH reduction.

Physiological concentration of GSH was also used to enhance the reactivation with and without the presence of RHLT. It was found that the increasing levels of GSH at 1, 2.5, and 5.0 mM could enhance the recovery to 176%, 257%, and 347% above the damaged enzyme activity. However, the presence of RHLT could correspondingly restore more of the GPx activity to 250%, 333%, and 440% over the cystine-inactivated enzyme activity (data not shown).

**Regeneration of G-3PD Activity in Rabbit Epithelial Cells by RHLT.** The highly oxidative, stress-sensitive G-3PD in rabbit lens epithelial cells showed nearly 80% loss in activity within 15 minutes after the cells were exposed to a bolus of 0.5 mM H$_2$O$_2$. The activity was recovered up to threefold when RHLT was added to the reaction mixture (Fig. 6). DTT effectively reactivated G-3PD, but GSH showed little effect under the same conditions. In a separate experiment when physiological level of GSH was used with and without the presence of RHLT, again, the activity of G-3PD restored more when RHLT enzyme was present. The restoration was from 7.5% to 19%.

**Table 2. Oxidation of RHLT by H$_2$O$_2$ and Its Regeneration**

<table>
<thead>
<tr>
<th>Samples/Treatments</th>
<th>% Activity of Reduced RHLT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivated RHLT (0.33 mM H$_2$O$_2$)</td>
<td>39 ± 8.5</td>
</tr>
<tr>
<td>Inactivated RHLT + GSH (0.5 mM)</td>
<td>57 ± 10</td>
</tr>
<tr>
<td>Inactivated RHLT + GSH (5 mM)</td>
<td>86 ± 14.1</td>
</tr>
<tr>
<td>Inactivated RHLT + DTT (5 mM)</td>
<td>98 ± 2.1</td>
</tr>
<tr>
<td>Inactivated RHLT + TRx/TR</td>
<td>91 ± 6.4</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 3. TRx/TR, thioredoxin/thioredoxin reductase system.
and 30% at GSH concentrations of 1.0 and 5.0 mM, respectively. 

DISCUSSION

The human lens TTase cDNA was found to be identical with the sequence from other tissues, as reported by Christensen et al.,16 Padilla et al.,13 and Park and Levine.41 The N-terminal amino acid sequence of the purified RHLT was the same as its sequence from other tissues, as reported by Christensen et al. and 30% at GSH concentrations of 1.0 and 5.0 mM, respectively.

The presence of RHLT, the recovery was increased from 6% to 39% and 56%, respectively (data not shown).

The human lens TTase cDNA was found to be identical with the sequence from other tissues, as reported by Christensen et al.,16 Padilla et al.,13 and Park and Levine.41 The N-terminal amino acid sequence of the purified RHLT was the same as its deduced sequence from cDNA and in agreement with TTase from other human tissue TTases. The purified enzyme showed physical and chemical properties similar to those of TTases from other sources.8,39,40 The catalytic function of RHLT for reducing HEDS was far more effective than GSH alone even when GSH was used at a physiological concentration. This suggests that participation of RHLT may be necessary to achieve an efficient thiol/disulfide exchange reaction. As shown in Table 1, the enzyme displayed a better catalytic efficiency for cystine than for the other two substrates, probably reflecting a steric and/or symmetrical influence. Substrate such as cystine with structural symmetry may expose its –S–S– bond more easily to the active site of TTase than do the asymmetrical ones.

The IAA-sensitive nature of RHLT is also similar to other TTases, indicating that the enzyme requires free thiols in the active center for activity. Inhibition by IAA could be easily protected after the enzyme was preincubated with S-sulfoxycysteine but not with GSH. This substrate protection can be considered as a mixed disulfide formation between TTase and S-sulfoxycysteine or intramolecular disulfide.2,59 thereby preventing irreversible SH alkylation by IAA. The partial inactivation of RHLT by H₂O₂ and its reactivation by reductants or by TRx–TR system indicates that the disulfide and free thiol interchange had occurred within the active site of the enzyme. This property has also been observed in nonocular TTases.42 RHLT appears to tolerate H₂O₂ quite well in comparison to other enzymes,29 because it still retained 30% to 40% activity even at concentrations 0.5 mM and higher. This apparent H₂O₂-resistant nature was observed more so in vivo with lens epithelial cells, in which TTase activity remained active in the H₂O₂-exposed cells even after 3 hours.29 Preliminary studies indicated that cellular TTase was induced when lens cells were exposed to H₂O₂, which may explain the sustained activity of TTase during oxidative stress. The fact that the TRx–TR system, another enzyme in the TDOR family known for its ability to reduce protein–protein disulfides,44,45 protected RHLT from in vitro oxidation may further explain the resistance of TTase to H₂O₂ inactivation in vivo.29

Although the wide distribution of TTase is well known, its physiological role is still not established.2 Because TTase can catalyze the reduction of protein–thiol mixed disulfides and low-molecular-weight disulfides in the presence of GSH,46,47 it has been implicated to play a role in many biological processes, from reductive denaturation of insulin, homeostatic maintenance of intracellular thiols, and modulation of enzyme and receptor activities to gene expression.1,17 The successful cloning and overexpression of the human lens TTase gene allowed us to obtain homogeneous RHLT for functional studies.

The total amount of PSSG and PSSC in a normal lens from animals and humans has been consistently lower than 5% of the free GSH pool,48 but the concentration can be elevated precipitously in experimental lenses exposed to oxidative stress47 or in lenses extracted from cataract patients.49 The interesting finding that elevated PSSG in a lens preexposed to an oxidant could spontaneously return to its normal level once the oxidant was removed25,26,28,50 prompted our speculation that TTase may involve in situ to control the rise of intracellular pool of S-thiolated proteins and to refrain the proteins from disulfide formation so that a proper lens function can be preserved.51

The present study provided further proof that lens TTase may also protect key metabolic enzymes from permanent oxidative damage. RHLT showed an ability to regenerate oxidant-inactivated enzyme activities of GPx, an important lens oxidation defense enzyme, and GST, a key xenobiotic detoxifying enzyme in the lens. Both enzymes were inactivated by cystine thiolation to form enzyme-cysteine mixed disulfide (PSSC). Interestingly GST could not be inactivated by GSH thiolation, as reported by Terada et al.52 and by Mieyal et al.4 Our observation paralleled these findings (result not shown). The reactivation of S-thiolated GST, GPx, or G-3PD could only be efficiently achieved by the catalytic function of RHLT and not by simple GSH reduction, even when physiological levels of GSH (1.0–5.0 mM) were used. The ability of RHLT to dethiolate PSSC in modified GST or GPx is in agreement with the finding of Terada et al. for placental TTase. This is in contrast to the reports from Mieyal’s laboratory.2,53 in that RBC TTase showed a distinct preference for PSSG over PSSC. We also found that lens TTase preferentially dethiolated crystallin-S-S-glutathione, but it also showed some catalytic activity toward crystallin-S-S-cysteine when an radiolabel assay was performed.51,54 Further studies are needed to clarify this discrepancy.

The strongest evidence for the repair/protection function of TTase is that RHLT could reactivate the oxidatively damaged enzyme in situ, including cellular G-3PD, which is a key metabolic enzyme for lens ATP generation in the glycolytic pathway. The restoration of enzyme activity by RHLT dethiolation in vitro suggests that G-3PD in the rabbit lens epithelial cells preexposed to H₂O₂ was likely damaged by forming protein–thiol mixed disulfide. The current results enhanced our spec-
ulation in the previous report\textsuperscript{29} that the spontaneous recovery of cellular G-3PD at the end of 3 hours, after the cells detoxified the bolus H$_2$O$_2$ (0.5 mM), may be attributed to the in situ repairing or dethiolating function of the oxidant-resistant TTase in the same cells. This speculation is in agreement with the report of the H$_2$O$_2$-treated human endothelial cells, in which a major S-thiolated protein was identified as G-3PD.\textsuperscript{55}

These and our previous studies indicate that lens TTase not only could dethiolate S-thiolated proteins, but also could generate enzyme activities by dethiolating the S-thiolated enzymes in the lens. The possible physiological function of TTase in the lens can therefore be considered as maintenance of the homeostasis of cellular thiol/disulfide equilibrium, thus protecting the lens from permanent oxidative damage by repairing the thiolated proteins or enzymes.

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References


