Cloning, expression and characterization of human thioltransferase (glutaredoxin) in *E. coli****

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Abstract PCR primers were designed from the known amino acid (aa) sequence for human red blood cell thioltransferase (hRBC TTase) and the known cDNA sequence for pig liver TTase (82% homologous) and used to amplify thioltransferase from a pool of human brain cDNAs. The PCR product was inserted into the pKK233-2 expression vector. The DNA sequence of the insert agreed with the aa sequence. High level expression of the enzyme was accomplished in *E. coli*, and Western blot analysis confirmed its identity. Recombinant TTase displayed catalytic properties indistinguishable from natural hRBC TTase.

Key words: Thioltransferase; Glutaredoxin; Cloning; Sulfhydryl

1. Introduction

Thioltransferase (TTase), also known as glutaredoxin, is a member of the thiol-disulfide oxidoreductase (TDOR) family which also includes thioredoxin and protein disulfide isomerase. These enzymes are believed to be involved in regulating sulfhydryl homeostasis through catalysis of thiol-disulfide interchange reactions [1–4]. The environment inside cells is predominantly reducing due to high levels of reduced glutathione (GSH); however, oxidative stress can alter this environment allowing exposed cellular thiols to become oxidized. Oxidation of exposed protein thiols can lead to protein inactivation [1,4], and it has been postulated that TDOR enzymes function to protect or repair sensitive sulfhydryls in times of oxidative stress [1–4].

TTase is a small (MW = 11.7 kDa) cytosolic protein that displays a high degree of amino acid sequence homology among mammalian species, and it is selective for glutathione-containing mixed disulfide substrates [1,5]. The actual physiological functions of TTase remain unresolved, and investigation of these functions would be greatly facilitated with recombinant enzyme technology. Here we report the cloning, expression and characterization of recombinant human TTase.

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2. Materials and methods

2.1. Materials

Glutathione and glutathione disulfide reductase were purchased from Sigma. NADPH was obtained from Boehringer Mannheim. S-sulfocysteine and L-cysteinyl-glutathione were purchased from Toronto Research Chemicals, Inc. DNA primers were synthesized in a 380B or 394 DNA synthesizer from Applied Biosystems. Taq Polymerase, T4 DNA ligase, Klenow, restriction enzymes, and subcloning efficiency DH5α cells were from Gibco BRL. Pfu polymerase was from Stratagene. Sephadex G-75 gel filtration medium was obtained from Pharmacia. The micro BCA protein assay was obtained from Pierce. ISS Pro-Blue system was purchased from Integrated Separation Systems. Wizard mini and maxi prep kits were from Promega, and the Qiaex DNA purification system was purchased from Qiagen. All other chemicals were at least reagent grade from standard sources.

2.2. Cloning of cDNA

Primers were designed based on the amino acid sequence of hRBC TTase that we determined previously [6] and the cDNA sequence for pig liver TTase [7] which has 82% amino acid sequence homology [6,8]. *Nco*I and *Hin*dIII restriction sites were added to the ends of the 5′ and 3′ primers, respectively, to enable easy insertion of the TTase cDNA into pKK233-2. This vector was chosen because previously it was shown to direct high level expression of pig liver TTase [9]. The synthesized primers were used in the polymerase chain reaction (PCR) to amplify TTase cDNA from the template, a pool of human brain cDNAs. The human brain cDNA was obtained from reverse transcription reactions with random primers and RNA purified from the cerebral cortex of several human brains.

2.3. Plasmid analysis

The ligated vector was transformed into DH5α cells, and several clones were chosen for analysis. The presence of the TTase cDNA insert was verified by restriction digestion analysis and resulting positive clones were designated pCC-TT1 and pCC-TT2.

2.4. Sequence analysis

Sequences of the CC-TT1 and CC-TT2 inserts in both the 3 and 5 prime directions, were obtained by automated fluorescence DNA sequencing, in the core facility of the Cleveland Clinic Foundation.

2.5. Expression of recombinant thioltransferase

Expression of TTase from pCC-TT1 (or pCC-TT2) was done in DH5α *E. coli* cells. Cells in log phase were treated with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and grown for an additonal 6 h. The cells were centrifuged (10,000 rpm in a JA-20 rotor for 30 min), resuspended in 10 mM sodium phosphate, pH 7.5 and then lysed by passage through a French Press. The lysed cells were centrifuged to remove cell debris and the supernatant was concentrated by passage through an Amicon ultrafiltration device with a YM3 filter. The concentrated sample was purified using a Sephadex G-75 column (2.5 × 130 cm) equilibrated and eluted with 10 mM sodium phosphate, pH 7.5 (see section 3).

2.6. Analysis of purified recombinant thioltransferase

The enzymatic activity of recombinant TTase was analyzed using spectrophotometric and radiolabel assays as described previously [5,10]. In addition, the radiolabel assay was done with two different mixed disulfide substrates [^{35}S]BSA-SSCysteine and [^{35}S]BSA-SSG,

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which were prepared as described previously [5]. The two different substrates were used to verify the substrate selectivity of the recombinant enzyme relative to natural hRBC TTase. The amount of protein in various samples was quantified using the BCA assay according to manufacturer's instructions (Pierce). SDS-PAGE and Western blot analyses were performed as previously described [11].

3. Results

3.1. Cloning

TTase was amplified using primers directed against the known aa sequence of hRBC TTase and the cDNA sequence of pig liver TTase. This amplified cDNA was purified, digested, and ligated into pKK233-2 (Fig. 1) to create the pCC-TT1 (pCC-TT2) plasmid. The result was verified by restriction digest analysis with *Xho*I (unique to the TTase insert), *Nco*I and *Hin*dIII. The insert from the original TTase clone was sequenced, and it corresponded to the amino acid sequence that we reported previously [6], except for a single amino acid difference at position 55 (tyrosine instead of asparagine). Since the known amino acid sequence [6] and the cDNA sequence of brain TTase recently published by Fernando et al. [12] contained asparagine at this position and because the change was caused by a single base difference in the codon, it was interpreted as an error by taq polymerase. Therefore we designed new primers to mutate the altered base back to wild type. Pfu polymerase was used instead of taq in these PCR reactions to ensure greater fidelity during elongation. Fig. 2 shows the cDNA sequence obtained from this second clone pCC-TT2 which corresponds exactly to the amino acid sequence that we determined previously [6] and which was confirmed by Padilla et al. [8]. The cDNA reported by Fernando et al. [12] differs from both our sequence and that of Padilla et al. [8] by a valine instead of leucine at position 96. Since the leucine at position 96 is well conserved throughout mammalian species [1], it is interpreted as wild type and was not changed. The TTase insert has also been removed from the pKK233-2 parent vector and inserted into pBluescript, as well as into the mammalian expression vector pREP10.

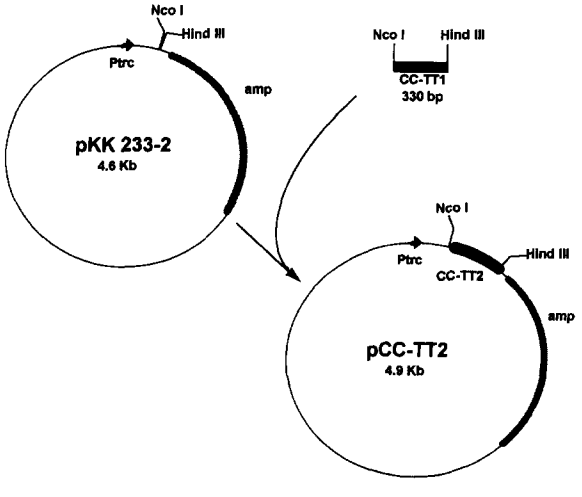

Fig. 1. Cloning strategy for recombinant human thioltransferase.

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          10          20          30          40          50
ATG GCT CAA GAA TTT GTC AAC TGC AAA ATC CAG CCT GGG AAG GTG GTT GTG TTC
Met Ala Gln Glu Phe Val Asn Cys Lys Ile Gln Pro Gly Lys Val Val Val Phe

     60          70          80          90          100
ATC AAG CCC ACC TGC CCG TAC TGC AGG AGG GCC CAA GAG ATC CTC AGT CAA TTG
Ile Lys Pro Thr Cys Pro Tyr Cys Arg Arg Ala Gln Glu Ile Leu Ser Gln Leu>

110         120         130         140         150         160
CCC ATC AAA CAA GGG CTT CTG GAA TTT GTC GAT ATC ACA GCC ACC AAC CAC ACT
Pro Ile Lys Gln Gly Leu Leu Glu Phe Val Asp Ile Thr Ala Thr Asn His Thr>

         170         180         190         200         210
AAC GAG ATT CAA GAT TAT TTG CAA CAG CTC ACG GGA GCA AGA ACG GTG CCT CGA
Asn Glu Ile Gln Asp Tyr Leu Gln Gln Leu Thr Gly Ala Arg Thr Val Pro Arg>

     220         230         240         250         260         270
GTC TTT ATT GGT AAA GAT TGT ATA GGC GGA TGC AGT GAT CTA GTC TCC TTG CAA
Val Phe Ile Gly Lys Asp Cys Ile Gly Gly Cys Ser Asp Leu Val Ser Leu Gln>

          280         290         300         310         320
CAG AGT GGG GAA CTG CTG ACG CGG CTA AAG CAA ATT GGA GCT CTG CAA TAA
Gln Ser Gly Glu Leu Leu Thr Arg Leu Lys Gln Ile Gly Ala Leu Gln ***>
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Fig. 2. DNA sequence and corresponding amino acid sequence of thioltransferase [6].

3.2. Expression

Yields of TTase from several DH5α *E. coli* preparations have ranged between 3–13% of total cytosolic protein. Some preparations indicated that the effect of IPTG is variable, but this was not studied separately. The specific activity of recombinant TTase obtained from several preparations after purification by gel filtration chromatography has ranged from 60–104 units/mg, corresponding to 54–94% purity by comparison to the specific activity of pure hRBC TTase [10]. The relative purity was confirmed by SDS-PAGE analysis (Fig. 3), where a single characteristic band which corresponds to the molecular weight of TTase was observed in the sample of purified protein from lysates of bacteria containing pCC-TT2; no such band appeared upon SDS-PAGE analysis of purified protein from lysates of bacteria transformed with pKK233-2 (plasmid without TTase inserted). Western blot analysis with antibodies to hRBC TTase revealed immunoreactive bands at the appropriate molecular weight (Fig. 4).

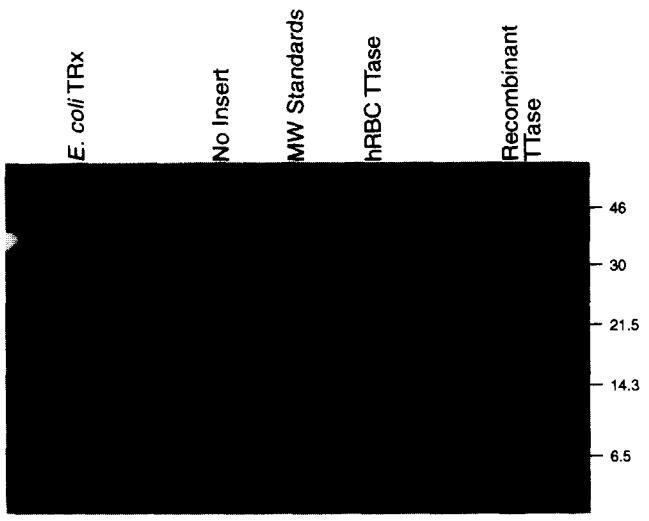

Fig. 3. Analysis of purified recombinant human thioltransferase. SDS-PAGE gel (6% stacking, 15% gel) was run for approximately 1 h at 5 mA and developed with ISS Pro Blue stain, as directed by the manufacturer. Lane 1: 1.5 μg thioredoxin; lane 2: no sample; lane 3: 2 μg of purified, concentrated protein from cells with pKK233-2 (no insert); lane 4: molecular weight standards kDaltons; lane 5: 1.5 μg hRBC TTase; lane 6: no sample; lane 7: 2 μg of purified, concentrated protein from cells with pCC-TT2 (TTase insert).

3.3. Catalytic characteristics

The recombinant and natural TTase were initially compared using the spectrophotometric assay, and their activities were indistinguishable. Further, using the spectrophotometric assay with recombinant TTase and hRBC TTase at equivalent enzymatic activities, the dependence of the rates of disulfide reduction on substrate concentration were indistinguishable (Fig. 5). Moreover, as previously reported for hRBC TTase [5], recombinant TTase catalyzed dethiolation of glutathione-mixed disulfides only (Table 1). Thus, the catalytic characteristics of recombinant TTase were indistinguishable from natural hRBC TTase both with respect to substrate selectivity and kinetic characteristics.

4. Discussion

This work reports the cloning, expression, and characterization of human TTase. The protein was purified using a simple procedure that produces TTase faster, and at higher yields than isolation of the natural hRBC enzyme [10]. Two other reports on the cDNA sequence for human TTase have appeared, but expression and characterization of the enzyme were not reported [8,12]. However more recently and simultaneously with our preliminary presentation of the current data [13] at the 1995 ASBMB/ACS meeting in San Francisco the cloning and expression of human placental TTase was presented by Meyer and Wells [14].

TTase is thought to be involved in a variety of physiological functions including enzyme regulation, and cellular protection [1–4]; and further it has been postulated to be involved in signal transduction [1,3]. Glutathionylation of proteins has been observed in a variety of different cells [15–19], and it has been postulated that TTase may catalyze both the formation and breakdown of glutathionylated proteins. The function of protein glutathionylation is not yet known, however, it is possible that glutathionylation acts to protect cellular proteins by reversibly binding to sensitive sulfhydryls during times of oxidative stress [1]. Recently was reported [20] that TTase levels are lower in a variety of chemically transformed cells when compared to the untransformed cell type, suggesting that variations in TTase levels may have important cellular consequences. The creation of mammalian cell lines that over- and under-express TTase will provide an experimental paradigm in which the cellular functions of TTase can be studied more directly, including the effect TTase has on protein glutathionylation. In addition, high levels

Table 1
Specificity of natural and recombinant thioltransferase

Source of thioltransferase	Enzyme-mediated dethiolation of BSA-S-SG[a] nmol [^{35}S]SG/min/nmol TTase	Enzyme-mediated dethiolation of BSA-S-SCys[b] nmol [^{35}S]SC/min/nmol TTase
hRBC TTase	332 ± 13 min^{-1} [c]	None Detected
Recombinant	328 ± 26 min^{-1} [d]	None Detected

Release of glutathionyl ([^{35}S]SG) or cysteinyl ([^{35}S]SC) equivalents from the labeled BSA mixed disulfide substrates was measured using the radiolabel dethiolation assay [5]. Non-enzymatic rates were subtracted from the overall rates.
[a] Reactions were done at 30°C and contained 0.5 mM GSH, 0.1 mM [^{35}S]BSA-SSG and several concentrations of TTase (8–50 nM), at pH = 7.5.
[b] Reactions were done at 30°C and contained 1 mM GSH, and 0.5 mM [^{35}S]BSA-SSCysteine and several concentrations of TTase (8–50 nM), at pH = 7.5.
[c] Value represents average of 9 experiments ± standard error.
[d] Value represents average of 7 experiments ± standard error.

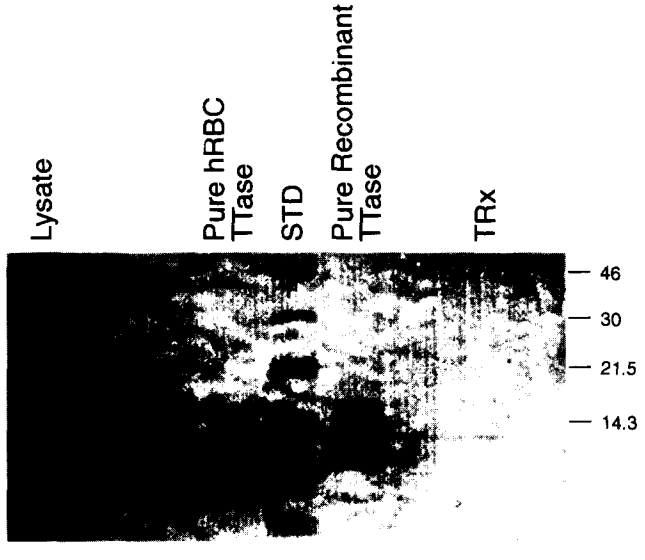

Fig. 4. Western blot analysis of recombinant human thioltransferase. SDS-PAGE gel treated the same as described under Fig. 3 except that the gel was transblotted to nitrocellulose for 45 min at 1 A prior to developing. The nitrocellulose blot was treated with anti-TTase [11], and a horseradish peroxidase conjugated secondary antibody, and then developed with the chromogenic substrate 4-Chloro-1-Naphthol. Lane 1: 1 μg of TTase in concentrated pre-column lysate; lane 2: no sample; lane 3: no sample; lane 4: 1.5 μg hRBC TTase; lane 5: molecular weight markers; lane 6: 1 μg purified, concentrated recombinant TTase; lane 7: no sample; lane 8: 1.5 μg *E. coli* thioredoxin.

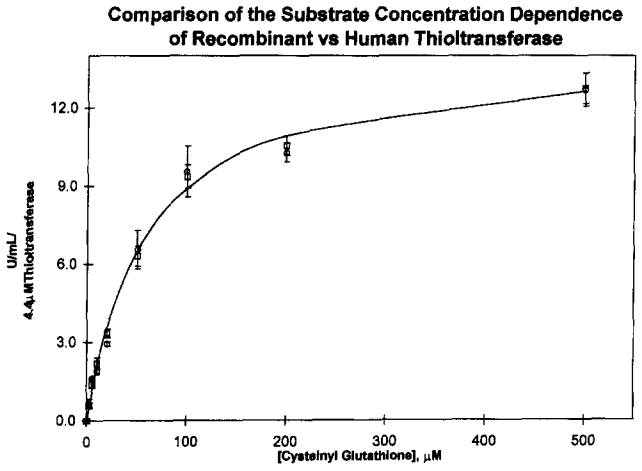

Fig. 5. Substrate concentration dependence for natural and recombinant thioltransferases. Reactions contained 0.2 mM NADPH, 0.5 mM GSH, 2 units/ml GSSG reductase in 0.1 M potassium phosphate, pH 7.4, and initiated with 2–500 mM cysteinylglutathione, in a total volume of 0.2 ml. ○ recombinant TTase; □ human red blood cell TTase. Rates were measured as time dependent changes in absorbance at 340 nm. The rates of non-enzymatic reactions were subtracted, and the difference plotted against cysteinylglutathione concentration.

of pure, wild type, and eventually mutant TTase protein will facilitate a variety of in vitro experiments, including structural studies with NMR.

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