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Cooperative kinetics of the recombinant glutathione transferase of *Taenia solium* and characterization of the enzyme

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ABSTRACT

Glutathione transferases (GSTs) are essential enzymes in many organisms due their diverse functions and, in helminths they are the main detoxification system. For *Taenia solium*, two cytosolic GSTs with molecular masses of 25.5 and 26.5 kDa (Ts26GST) have been found. Ts26GST was cloned to be studied in its recombinant form (recTs26GST). Although the primary structure is related to the mu class, the kinetic parameters for CDNB (V_{max} = 51.5 μmol min^{-1} mg^{-1}; K_m = 1.06 mM; k_{cat} = 22.2 s^{-1}) are related with some alpha GSTs. The substrate and inhibitor class markers reaffirmed these bimodal characteristics. Inhibition studies with anthelminthics indicate that recTs26GST is sensitive to mebendazole, displaying a non competitive inhibition pattern suggesting that at least two molecules are binding to recTs26GST. On the other hand, the kinetic curves for CDNB and GSH showed a positive cooperativity that was corroborated using fluorometric assays. Those assays indicate that CDNB binding is highly influenced by GSH, probably by modulation of the Ts26GST conformational ensamble.

Glutathione transferases (E.C. 2.5.1.18; GSTs)[1] constitute a versatile family that detoxifies a wide spectrum of electrophilic compounds by glutathione (GSH) conjugation. They also participate in other processes such as leukotriene and prostaglandin biosynthesis, transport of molecules, regulation in signal and transcription processes, isomerizations, catabolism of aromatic amino acids and modulation and/or formation of ion channels [1–6]. They participate in the second phase of the detoxification system and are localized in microsomes, mitochondria, and cytosol. The cytosolic GSTs (cGSTs) are recognized as ubiquitous enzymes grouped in the mu (M), alpha (A), pi (P), theta (T), sigma (S), zeta (Z), and omega (O) classes, although there are also organism-specific classes, such as nu (nematode), lambda, phi and tau (plants), beta (prokaryotes), delta, epsilon, iota and xi (insects). This classification has been based on the biochemical, genetic, immunological and structural properties of the GSTs [4,6–8]. In helminths, this family is especially important in oxidative stress control because helminths lack CYP450 activity [9], and so it is probable that the metabolism of many xenobiotics, ecosanoids and other typical CYP450 substrates dealt with by the GST family [10]. In general, cGSTs are dimers with two active sites and contain 220 amino acids on average. Each active site is composed of two domains: the first is the G-site in the N-terminal that binds and activates the GSH, and the second is the C-terminal H site, which binds the hydrophobic compound and is considered as a variable region responsible for the broad range of substrates that can be conjugated [1,6]. Although it has been demonstrated that cGSTs monomers can act independently, these enzymes have activity only as dimers *in vivo* [11]. This behavior was used to postulate the existence of a cooperative process between subunits influenced by GSH and substrate binding. This hypothesis has been confirmed recently in some mammalian GST subclasses and in *Plasmodium falciparum* [12,13].

Taenia solium, classified as a potentially eradicable parasite by WHO in 1993, causes the common parasitoses called cysticercosis and taeniosis. The first is the main neurological disease of parasitic origin associated with epilepsy in humans and causing great morbidity on a global scale [14,15]. Despite efforts to eradicate the parasite with vaccination in pigs and massive chemotherapy using anthelminthics in humans and swine, a conclusive control strategy has not been achieved [16–20]. In recent years, there have been an increased number of reports about drug resistance in parasites, some of them related to GSTs [21,22]. Therefore, new approaches are needed to overcome this problem, and one option would be to inhibit enzymes vital to the parasite, such as the GSTs [23].

In this cestode, two cGSTs with molecular masses of 25.5 and 26.5 kDa [24] have been identified. The N-terminal sequences of both cGSTs showed a close relation with the M-class [25]. Subsequent characterization of the recombinant GST 25.5 kDa (Ts25GST)

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[1] *Abbreviations used:* GSTs, glutathione transferases; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; BSF, bromosulfophthalein; DCNB, 1,2-dichloro-4-nitrobenzene; ETA, ethacrynic acid; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy) propane; CHP, cumene hydroperoxide; CB, cibacron blue; TFC, triphenyltin chloride; MBZ, mebendazole; SOBM, super optimal broad medium; IPTG, isopropyl β-D-thiogalactopyranoside; ORF, open-reading frame.

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confirmed its M-class characteristics [25], but biochemical analysis of the native GST 26.5 kDa (Ts26GST) purified from a cysticerci soluble extract suggested that its biochemical properties were a composite of those characteristic of the GST M and A classes [26]. To confirm this behavior of Ts26GST, we produced and biochemically characterized the recombinant enzyme (recTs26GST) and found new catalytic mechanism for a cestode enzyme.

Methods and materials

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), bromosulfophthalein (BSF), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (ETA), 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP), cumene hydroperoxide (CHP), alkenals like trans-2-nonenal and hexa-2,4-dienal, the inhibitors cibacron blue (CB), triphenyltin chloride (TFC), and the anthelminthics albendazole, mebendazole (MBZ), thiabendazole, and praziquantel were purchased from Sigma–Aldrich (ST. Louis, MI, USA). *Escherichia coli* strains C600hfl, TOP10, JM105, endonucleases NcoI, BglII, and pCRII-TOPO vector (TOPO TA Cloning kit Dual Promotor) were acquired from Invitrogen (Carlsbad, CA, USA). The nylon and nitrocellulose membranes (Hybond), the Thermo Sequenase Cy5.5 Dye Terminator Sequencing Kit, forward and reverse M13 primers and ^{32}P-dCTP isotope were purchased from Amersham Bioscience (Buckinghamshire, UK). Other reagents of high analytical grade were obtained from local sources.

Generation of the GST26.5 probe

The primers GST26F (5′-AAG-TAC-AAG-TTC-GCN-TAY-TGG-AA, sense) and GST26R (5′-ATG-AGG-TAN-GGN-AGR-TTN-GG, antisense) were designed based on the following underlined amino acids from the Ts26GST sequence obtained by Edman degradation of the N-terminus (MNKYKFAYWNLRGLGDQIRLILEF) [25] and from an internal sequence (TDLGFDFPNLPYLIDGDK) of a trypsin-generated peptide of the enzyme. Using these primers in PCR reactions, an 200 bp amplicon was obtained, purified, cloned, and sequenced. This DNA segment, coding for a region in the N-terminal domain, was labeled with ^{32}P-dCTP using a RedPrime kit (Amersham) and used as a probe.

cDNA library screening, sequence analysis, and cloning of the Ts26GST cDNA

Approximately 15,000 phages from a λgt10 cDNA library of *T. solium* larvae [27] were screened in *E. coli* C600hfl as host, cultivated in super optimal broad medium (SOBM). Phages were transferred to nitrocellulose membranes and denaturalized (0.5 M NaOH, 1.5 M NaCl), neutralized (1 M Tris–HCl, 1.5 M NaCl, pH 8) and washed (SSC2X). The screening of the cDNA library was performed as previously described [25]. The cDNA fragments of positive clones were amplified by PCR. The larger fragments were cloned in pCRII-TOPO vector and sequenced using the Thermo Sequenase Cy5.5 Dye Terminator Sequencing Kit and the M13 primers on an automated SEQ4X4 sequencer (Amersham Pharmacia Biotech, UK). The cDNA sequences were analyzed with the bioinformatics programs BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) CLUSTALW (http://www.ebi.ac.uk/clustalw/), and ExPASy (http://www.expasy.org/).

Expression and purification of the recombinant enzyme

The Ts26GST cDNA coding region was amplified by PCR with the primers GSTNco (5′-CAT-GCC-ATG-GAT-AAG-TAC-AAA-TTC-GCC) and GST27-X2 (5′-GGA-AGA-TCT-TCC-TTA-TCT-TTT-TTT-AGG-GG), which contained NcoI and BglII sites on their 5′ and 3′ ends, respectively. The amino acids MNKY, obtained by Edman degradation, were added to the 5′ primer GSTNCO to amplify the complete coding region of the enzyme by RT-PCR using mRNA from the parasite. The fragment obtained was ligated into plasmid pCRII-TOPO and verified by sequence analysis. This plasmid was then digested with NcoI and BglII and the Ts26GST fragment subcloned into the pTR99A expression plasmid digested with the same enzymes (Amersham Pharmacia Biotech). This expression construct was named GST26-pTR99 and used to transform *E. coli* strain JM105.

Transformed bacteria were grown at 37 °C overnight in 10 mL of Luria–Bertani (LB) medium supplemented with ampicillin (50 μg/mL). This culture was used to inoculate 500 mL of fresh LB-ampicillin (50 μg/mL) medium and grown at 37 °C to an OD_{600nm} of ∼0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 2 mM and the bacteria were grown with stirring for 3.5 h at 37 °C. Bacteria were harvested by centrifugation at 1034*g* for 10 min and washed three times with PBS. The bacterial pellet was suspended in extraction buffer (0.15 M NaCl, 0.1 M potassium phosphate, pH 7.4, 1% triton, 0.5 M EDTA) and then lysed by sonication on ice for three periods of 1 min, with cooling for 2 min between each step. The suspension was centrifuged at 8804*g* for 20 min at 4 °C and the supernatant was diluted with column buffer (PBS containing 1% triton, pH 7.4) in a 1:2 ratio and filtered through of 0.45-μm membrane. The filtered supernatant was loaded onto a Sepharose–GSH column (equilibrated with a column buffer) and passed through it three times. The column was washed with PBS until no proteins were detected at 280 nm and the bound protein was eluted with 5 mM GSH in 50 mM Tris–HCl (pH 8.0). The eluted fraction was concentrated with an Amicon ultrafiltration system (Millipore, USA). Quantification of the recTs26GST was made by spectroscopy at 280 nm using an extintion coefficient of 38390 M^{-1} cm^{-1} obtained from its primary sequence (http://www.expasy.org/) [28], and by the Lowry method read at 600 nm [29]. The readings were performed with an Ultrospec *3100 pro* (Amersham Bioscience). Purity of recTs26GST was determined by SDS–PAGE in 12.5% resolving gels [30] followed by staining with Coomassie brilliant blue (Gibco-BRL).

Enzymatic assays

The recTs26GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) dissolved in 1.5% ethanol (v/v) as substrate ($\Delta\varepsilon$ = 9.6 m M^{-1} cm^{-1}), according to the method of Habig [31]. The reaction was followed by spectroscopy at 340 nm in the reaction buffer (0.1 M potassium phosphate, pH = 6.5, 1 mM GSH) for 3 min. The specific activity was calculated with the formula: $V\left(\frac{A2-A1}{\Delta\varepsilon(t2-t1)(\ell)(w)}\right)$ where V is the total volume of the reaction in mL, $A2$ and $A1$ are the absorbances at final and initial times $t2$ and $t1$ in sec, respectively, $\Delta\varepsilon$ is the extinction coefficient of the substrate (mM^{-1} cm^{-1}), ℓ is the path length of the cell (cm) and w is the quantity of protein (mg). Activities with ETA, BSF and alkenals (nonenal and hexadienal) were determined using Habig's procedure [31] as well. Activities of CHP were measured in a coupled assay as described by Lawrence [32] with 1.2 mM CHP, 1 mM GSH, 0.1 mM NADPH, and 1 U GSH reductase in 0.1 M potassium phosphate buffer (pH 7.0). The reaction was started by CHP addition and readings were made with an Ultrospec *3100 pro* at room temperature (23 ± 1 °C).

Kinetic parameters

Determinations of kinetic parameters were made as follows: in the first set of assays, GSH was maintained at 5 mM while the CDNB concentration was varied from 0 to 7.5 mM. In the second set of assays, the curve was constructed by fixing the CDNB concentration at 7 mM and changing the concentration of GSH from 0.1 to 6 mM. The assays varying CDNB concentrations were per-