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Characterization of catalytic and non-catalytic activities of EgGST2-3, a heterodimeric glutathione transferase from *Echinococcus granulosus*

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ABSTRACT

Glutathione transferases (GSTs) perform several catalytic and non-catalytic roles in the defense against toxicities of electrophile compounds and oxidative stress, and therefore are involved in stress-response and cell detoxification. Previously, we have provided evidence indicating that EgGST2 and EgGST3, two phylogenetically distant *Echinococcus granulosus* GSTs, can naturally form a heterodimeric structure (EgGST2-3). In the present work, the recombinant heterodimer GST (rEgGST2-3) is characterized. Hence, rEgGST2-3 was able to conjugate GSH to three substrates: 1-chloro-2,4-dinitrobenzene (CDNB, general substrate for GSTs), 1,2-dichloro-4-nitrobenzene (specific substrate for mammalian Mu class) and *trans,trans*-deca-2,4-dienal (reactive carbonyl). The canonical activity was considerably reduced by all the conventional inhibitors (cybacron blue, triphenylthin chloride and bromosulfophthalein) and by other inhibitors (ellagic acid, alizarin and chenodeoxycholic acid). Besides this, rEgGST2-3 activity was inhibited by a number of anthelmintic drugs, where the halogenated phenolic drugs (mainly bithionol and hexachlorophene) acted as stronger inhibitors, suggesting they may bind to the EgGST2-3. Moreover, rEgGST2-3 exhibited glutathione-peroxidase activity, and its specific constant (k_{cat}/K_M) was calculated. Finally, rEgGST2-3 displayed the ability to bind non-substrate molecules, particularly anthelmintic drugs, suggesting that ligandin activity may have potential to act as a passive protection parasite mechanism. Overall, the rEgGST2-3 behavior was shown to be both complementary and redundant to that reported for rEgGST1, another characterized GST from *E. granulosus*. It would be appropriate that different enzymes in the same organism do not have exactly the same functional properties to develop a better adaptation to life in the host.

1. Introduction

Hydatid disease is a zoonosis caused by infection with the larval stage of the dog tapeworm *Echinococcus granulosus*. In intermediate host, several livestock species and humans, the metacestode slowly grows into a fluid-filled bladder (hydatid cyst) at the viscera, mainly liver and lung. Mature metacestode produces numerous protoscoleces, which are the dog infective forms. This parasitic disease occurs worldwide causing important health and economic problems (Eckert and Deplazes, 2004; Craig and Larrieu, 2006).

Glutathione transferases (GSTs, EC 2.5.1.18) are a ubiquitous superfamily of multifunctional proteins which are part of “phase II” enzymes in cellular metabolic detoxification process. They are involved in clearance of cytotoxic and genotoxic compounds and in protection against oxidative damage (Hayes and Pulford, 1995; Sheehan et al., 2001). GSTs catalyze the nucleophilic addition of the thiol of reduced glutathione (GSH, gamma-glutamyl-cysteinyl-glycine), to a wide range of exogenous and endogenous hydrophobic electrophiles. Moreover, they can act non-enzymatically, through binding to non-substrate ligands and other reactive compounds, which facilitate their elimination (Wilce and Parker, 1994). In mammals, cytosolic GSTs can be grouped into at least seven different classes (Alpha, Mu, Pi, Theta, Sigma, Zeta and Omega) according to their amino acid sequences (Pearson, 2005), where the proteins in a same class typically share at least 40% sequence identity whereas members of different classes share less than a 25% (Hayes et al., 2005). The three-dimensional structure of the polypeptide chain seems to be conserved with in all GSTs where two domains can be identified: the highly conserved *N*-terminal domain and the less conserved *C*-terminal domain. The former, similar to the thioredoxin domain, contains the GSH binding site (G-site), while the latter contains the hydrophobic-substrate binding site (H-site) (Frova, 2006). Although all cytosolic GSTs share similar shape, differences between them lead to

Abbreviations: GSTs, glutathione transferases; CDNB, 1-choro-2,4-dinitrobenzene; GSH, glutathione; EgGST, *E. granulosus* glutathione transferase; rEgGST, recombinant EgGST; GPx, GSH peroxidase activity; DHA, dehydroascorbate

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class-specific characteristics of their enzyme activity. For example, cytosolic GSTs described to date are generally active as dimers (Frova, 2006) and the dimerization regions are conserved within the members of a same GST class (Frova, 2006; Board and Menon, 2013). In particular, conserved residues have been described for this region that allow the dimerization of proteins from the same class (Hayes and Pulford, 1995), but which render dimerization of the interclasses subunits incompatible. Moreover, the catalytic residue at the G-site (ie Tyr, Ser and Cys for Sigma, Theta and Omega classes respectively) as well as its location within the folded structure causes differences in their catalytic mechanisms (Atkinson and Babbitt, 2009).

In *E. granulosus*, we have identified three phylogenetically distant cytosolic GSTs, EgGST1, 2 and 3 (Fernández et al., 2000; Iriarte et al., 2012), where EgGST1 and EgGST2 were classified as Mu and Sigma respectively. In contrast, EgGST3 seems to belong to a new GST class, since it phylogenetically lies in-between Sigma and Omega classes (Iriarte et al., 2012). Recently, we have provided evidence indicating that EgGST2 and EgGST3 can naturally form a heterodimeric structure (EgGST2-3), and the resulting stable recombinant protein displayed GST activity (Arbildi et al., 2017). In this context, as a follow-up of our studies, here we report the biochemical characterization of the recombinant protein (rEgGST2-3), which includes catalytic and non-catalytic properties as well as inhibitor and substrate specificities. The data obtained may be useful from a physiological perspective in which the EgGST2-3 enzyme is a multifunctional protein with a range of potentially interesting catalytic and binding activities. In particular, it could play a role in protection against host-derived lipid peroxidation and in the passive detoxification of anthelmintic drugs.

2. Material and methods

2.1. Expression and purification of rEgGST2-3

Expression of the recombinant heterodimer protein (rEgGST2-3) in a prokaryote system was done as described previously (Arbildi et al., 2017) with few modifications. Briefly, rEgGST2-3, obtained by co-expressing of both subunits (EgGST3 and His-tagged-EgGST2) in *Escherichia coli* BL21[DE3], was first purified using Ni-NTA Purification System (Invitrogen). Then, a gel filtration step in Superdex-75 (GE-Healthcare), equilibrated in 20 mM Tris-HCl buffer (pH 8) containing 150 mM sodium chloride was performed. The rEgGST2-3, containing N-terminal His-tag, was stored at 1 mg/mL (37 μM) in 20 mM Tris-HCl buffer (pH 8) containing 150 mM sodium chloride at − 20 °C until used. Enzyme molarity was always calculated considering the average molecular weight of the two monomers (27,000 g/mol).

2.2. Characterization of glutathione-conjugating activities of rEgGST2-3

In the following activities, each measurement was carried out in triplicates and at least two independent experiments were performed. The non-enzymatic reaction was always subtracted to the enzyme-catalyzed reaction. One activity unit was defined as the amount of enzyme catalyzing the turnover of 1 mol of the corresponding substrate per minute under the specified conditions

2.2.1. Standard catalytic assay

The canonic activity was checked under standard conditions as previously described (Arbildi et al., 2017) using 0.4 μM of rEgGST2-3, 4 mM GSH and 1 mM 1-choro-2,4-dinitrobenzene (CDNB). The reaction was initiated by adding the enzyme and readings were made with an Ultraspect 1000 spectrophotometer (Pharmacia Biotech). The final concentration of ethanol in the reaction mixture was always constant (0.9% v/v).

2.2.2. Substrate specificity

The ability of rEgGST2-3 (0.4 μM) to conjugate GSH to different second substrates was analyzed spectrophotometrically using rEgGST2-3 in an Ultraspect 1000 spectrophotometer (Pharmacia Biotech) as previously described (Harispe et al., 2010). The second substrates are listed in Table 1 (see assay conditions in Appendix A in Supplemtary material).

2.2.3. Inhibitor profile

A wide range of molecules (Table 2) were assessed for their capacity to inhibit rEgGST2-3 canonic activity, including conventional inhibitors and those described for some GSTs as well as several anthelmintic drugs. The assay was carried out as previously described (Harispe et al., 2010) with few modifications. Briefly, each molecule was incubated in a 96-well plate with 0.4 μM of rEgGST2-3 in 0.1 M phosphate buffer pH 6.5 containing 4 mM GSH, for 5 min at 25 °C. The reaction was then started by adding 1 mM of CDNB and monitored in a microplate reader FLUOstar OPTIMA (BMG Labtech). The sensitivity of rEgGST2-3 to each inhibitor was characterized by means of the IC_{50}, the concentration of inhibitor decreasing enzyme activity by 50%. This parameter was calculated graphically plotting initial rate as function inhibitor concentration and fitting to a sigmoidal function using a non-linear regression analysis. Mebendazole was only sparing soluble in the assay buffer; so the enzyme inhibition was determined at the highest concentration tested (200 μM).

2.3. Other catalytic activities of rEgGST2-3

GSH peroxidase (GPx) activity was assessed spectrophotometrically in a T70 + UV–vis (PG instruments Ltd) in a coupled reaction to the oxidation of NADPH at 30 °C as previously described (Harispe et al., 2010) with few modifications. Briefly, GPx activity was assessed using different rEgGST2-3 concentrations (0.8–4.4 μM) and 1 mM GSH, and the reaction was then initiated by the addition of cumene hydroperoxide (CHP, 1.2 mM). The final ethanol concentration in the assay solution was always 0.9% (v/v). One unit is defined as the amount of enzyme that will cause the oxidation of 1 μmol of NADPH to NADP + (which corresponds to a 1 μmol of CHP) per minute at 30 °C. In order to determine specificity constant (k_{cat}/K_M^{CHP}) for CHP, steady-state kinetic studies of the reduction of CHP by GSH were carried out as described in Danielson and Mannervik (1985) using various concentrations of CHP (0.5–3 mM) and GSH (0.5–5 mM) and fitting to the equation: $\{v/[rEgGST2\text{-}3] = (k_{cat}/K_M^{CHP})/[CHP]\}$. Thiol-transferase activity toward 2-hydroxyethyl disulfide (HEDS, 2 mM) and dehydroascorbate (DHA) reductase activity toward DHA (1.5 mM) were also assessed spectrophotometrically in a coupled reaction to the oxidation of NADPH as described in Schmuck et al. (2005). In all activities, the non-enzymatic reaction was subtracted to the enzyme-catalyzed reaction. For each assay, at least two independent experiments each performed in three replicates.

2.4. Ligand binding assays

Although the major functions of GSTs involve enzymatic catalysis, they can act as non-covalent binding proteins for a variety of hydrophobic ligands (Hayes et al., 2005). In order to determine the equilibrium dissociation constants, measurements of the intrinsic fluorescence of rEgGST2-3 (tryptophan, phenylalanine and tyrosine residues) following titrations with each ligand were performed as was described in Plancarte et al. (2014) with few modifications. Briefly, the measurements were carried out in 0.1 M phosphate buffer (pH 6.5) containing 0.1 M sodium chloride, at 25 °C using 3.1 μM rEgGST2-3 in a Varioskan Flash spectrofluorometer (Thermo scientific) in a 96 wells plate (0.2 mL final volume) with excitation and emission wavelengths of 280 and 340 nm, respectively. Different ligand concentration (see Table 3) was added and the reaction was started with the addition of the enzyme. The final concentration of DMSO or ethanol in the reaction mixture was always less than 10%. In all experiments, the buffer and