Drosophila GSTs display outstanding catalytic efficiencies with the environmental pollutants 2,4,6-trinitrotoluene and 2,4-dinitrotoluene

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1. Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) belong to a family of abundant phase II detoxication enzymes involved in the metabolism and inactivation of a broad range of structurally unrelated endogenous metabolites and xenobiotic electrophiles through glutathione (GSH) conjugation. Thereby, cells are provided with necessary protection against various cytotoxins [1–3]. GSTs have been found in almost all aerobic organisms from insects to plants to mammals, and even in many prokaryotes. On the basis of their amino acid sequences and structural similarities, the numerous soluble mammalian GSTs (also known as canonical or cytosolic GSTs) can be divided into seven different classes designated by their Greek names, alpha, mu, omega, pi, sigma, theta and zeta [4]. In insects six classes of soluble GSTs have been identified. The fruit fly Drosophila melanogaster contains omega, sigma, theta and zeta classes of GSTs, which appear to exist in almost all eukaryotes, plus two additional classes, delta and epsilon. The epsilon class is the most numerous class of soluble GSTs in Drosophila melanogaster represented by 14 GST genes [5].

GSTs exhibit broad substrate specificities towards various endogenous and xenobiotic electrophiles including aryl halides, α,β-unsaturated carbonyls, oxidized lipids, isothiocyanates, various drugs and pollutants [1,3,6,7]. Although many of these reactions are catalyzed by several different GSTs, each GST isoform shows its own substrate selectivity [2,8]. The nitroaromatic explosive 2,4,6-trinitrotoluene (TNT) and its precursor 2,4-dinitrotoluene (DNT) are important occupational and environmental pollutants introduced into nature by human activities. Many nitro-substituted explosives have been evaluated in laboratory studies and found to be toxic for almost all classes of organisms including algae, bacteria, plants and mammals [9].

Two plant tau class GSTs from Arabidopsis thaliana [10] as well as from poplar (Populus trichocarpa) along with an equine GST have been reported to have catalytic activities with TNT in vitro [11,12]. However no data have yet been reported showing human and insect GSTs catalyzing the conjugation of GSH with TNT and the closely related DNT (Fig. 1).

In the present investigation we have determined the catalytic activities with TNT and DNT displayed by a set of purified GSTs. Seven human GSTs from four different classes, namely, GSTA1-1 and GSTA2-2 (alpha class), GSTM2-2, GSTM4-4, and GSTM5-5 (mu class), GSTP1-1 (pi class), GSTS1-1 (sigma class), plus two GSTs from the Drosophila melanogaster epsilon class, DmGSTE6 and DmGSTE7. Further kinetic studies of the most active enzymes DmGSTE6 and DmGSTE7 were performed with both substrates.

2. Materials and methods

2.1. Materials

Unless stated otherwise, all chemicals used for enzymatic activity and kinetic measurements were purchased from...
The bacteria were grown overnight on LB-agar plates containing 50 cells were transformed by the electroporation technique and the 0.2 mM isopropyl-β-D-thiogalactopyranoside at an optical density of OD600 nm of 0.4. The bacteria were further allowed to grow at 37 °C for 16 h and cell pellets were kept at -80 °C until the purification was performed by Ni-IMAC as described previously [13]. Briefly, the pellets were dissolved in 25 ml of ice-cold buffer A (20 mM sodium phosphate buffer pH 7.8, supplemented with 85 mM imidazole, 500 mM NaCl, 10 mM β-mercaptoethanol, 0.02% NaN₃) and 0.2 mg/ml lysozyme, half a tablet of EDTA-free protease inhibitor (Roche Germany) and incubated for 30 min on an ice bath. The resultant suspension was lysed by sonication 5 × 20 s and centrifuged at 27,200g for 45 min at 4 °C. The cell debris was discarded and the supernatant containing the enzyme was incubated with pre-equilibrated Ni-IMAC gel on an ice bath for 30 min. The gel was packed into a column and the unbound proteins were washed away with buffer A. The bound enzyme was eluted with 500 mM imidazole (otherwise identical with buffer A) at a flow rate of 1 ml/min. The eluted fractions were pooled and dialyzed overnight against 10 mM Tris HCl buffer pH 7.8, containing 0.2 mM DTT and 1 mM EDTA. The other human GST isoenzymes were heterologously expressed in E.coli and purified by GSH-affinity chromatography as described by Kolm et al. [14]. The protein concentrations of the recombinant enzymes were determined by the Bradford assay [15] and the purity of the enzymes was assessed by SDS-PAGE. In order to verify that the purified GSTs were active, the standard GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) was used.

2.3. Enzyme activity assay

The enzymatic activities of the purified GSTs with 1 mM DNT or 0.2 mM TNT with 1 mM GSH were determined in 0.1 M sodium phosphate buffer, pH 6.5, at 30 °C for 30 min. The stock solutions of DNT were prepared in ethanol (resulting in 5% final ethanol concentration in 1 ml reaction mixture), while TNT stock was provided as 0.5 mM aqueous solution. After 30 min incubation the nitrite formation was assayed colorimetrically as described by French et al. [16] by using the Griess assay with modifications as follows. To a 360 μl of reaction sample, 360 μl of milli-Q H₂O and 180 μl of sulfanilamide (10 mg/ml in 0.68 M HCl) were added. The components were mixed well and incubated at room temperature for 10 min. After addition of 72 μl of N-(1-naphthyl)ethylenediamine (10 mg/ml in H₂O), and mixing and further incubation for 10 min the absorbance was measured spectrophotometrically at 540 nm. The nitrite released from the nitroaromatic compounds was quantified from a sodium nitrite standard curve with known concentrations. The blank reactions were prepared by using the same concentrations of the substrates without enzymes and the values were subtracted from the enzymatic reactions.

2.4. Kinetic measurements

Steady-state kinetic analyses of DmGSTE6 and DmGSTE7 with DNT and TNT substrates were performed with 3–13 μg enzyme in the assay under the same conditions as for the specific activity determinations. For the determination of kinetic parameter values, saturation curves were obtained by using at least seven different concentrations of DNT and TNT with a saturating GSH concentration of 5 mM. The reactions were linear for at least 30 min; < 6% of TNT and < 0.3% of DNT were consumed in that time under the conditions used. Nitrite formation was determined after 30 min by the Griess assay as described earlier. The TNT concentrations used were between 0.0125 and 0.4 mM, while DNT concentrations were varied from 0.025 to 5 mM. The concentrations of the TNT and TNT stock solutions limited the experiments to nonsaturating substrate levels.

2.5. Data analysis

All reactions for determination of both specific activities and the steady-state kinetic parameters were performed in triplicate. The nitrite formation for each reaction was quantified from a standard curve with known concentrations. The kinetic parameters values were obtained from GraphPad Prism 6.0 software by using non-linear regression analysis and the Michaelis–Menten equation. The kcat and kcat/Km values were calculated from the subunit concentrations of the enzymes used for the reactions.

3. Results

3.1. DNT and TNT as substrates for GSTs

To investigate the environmental pollutants and toxicants DNT and TNT as substrates for GSTs, a set of GSTs from the human alpha, mu, pi, and sigma classes as well as two epsilon class GSTs from D. melanogaster was used. Table 1 shows the catalytic activities of nine different enzymes with DNT and TNT measured under standard assay conditions in 0.1 M sodium phosphate buffer pH 6.5 at 30 °C. The temperature was chosen for comparison with previously published GST activities with other substrates at 30 °C. The DNT and TNT concentrations were below the corresponding Kₘ values and substrate saturation could not be obtained owing to limited solubility of the substrates in the assay system. Among the tested enzymes, the human GSTs were the least active with both substrates as compared to D. melanogaster DmGSTE6 and DmGSTE7, which showed higher specific activities. DmGSTE6 was the most active enzyme with TNT displaying a specific activity of 62.7 ± 2.6 nmol min⁻¹ mg⁻¹. DmGSTE7 showed a specific activity of 20.0 ± 2.0 nmol min⁻¹ mg⁻¹, which is 3-fold lower than that of DmGSTE6. However, the specific activities of DmGSTE6
(20.5 ± 1.4 nmol min⁻¹ mg⁻¹) and DmGSTE7 (14.3 ± 1.4 nmol min⁻¹ mg⁻¹) with DNT were more similar in magnitude. Among the different human GSTs, GSTA2-2 demonstrated the highest specific activity with DNT (2.6 ± 0.4 nmol min⁻¹ mg⁻¹), whereas < 0.01 nmol min⁻¹ mg⁻¹ activity was detected for this enzyme with TNT. By contrast, GSTM4-4 showed minute activity with TNT (0.09 ± 0.01 nmol min⁻¹ mg⁻¹) but < 0.01 nmol min⁻¹ mg⁻¹ activity with DNT. Other human GSTs showed comparatively moderate specific activities with both substrates. GSTS1-1 showed the lowest specific activities with both DNT and TNT (Table 1).

### 3.2. Steady-state kinetic parameters

Among all the tested enzymes DmGSTE6 and DmGSTE7 showed high specific activities with both DNT and TNT, and these enzymes were further characterized by determining their steady-state kinetic parameters. All the measurements were carried out at a saturating GSH concentration of 5 mM and by varying the concentrations of DNT and TNT (Fig. 2). Although rate saturation could not reached in the range of experimentally accessible substrate concentrations, the precision of the measurements allowed determination of the kinetic parameters by nonlinear regression analysis. The results summarized in Table 2 show that the catalytic efficiency (kcat/Km) of DmGSTE6 with TNT is almost 3-fold higher than that of DmGSTE7, while the kcat values were not significantly different. With DNT as substrate both the kcat and Km values were approximately two-fold higher for DmGSTE6 than for DmGSTE7. As a result, the catalytic efficiencies of both the enzymes were similar.
A study of enzymes that evolved via multiple gene duplications to diverse classes of mammals and insects remains largely unexplored. However, detoxication of TNT and related compounds in nitroradical that promotes formation of reactive oxygen species in the soil and surface waters due to military and industrial activities produced toxic compounds as well as the man-made chemical pollutants, which in turn pose an ever increasing challenge to the ecological system. Due to their chemically resistant structures, a great number of man-made chemicals such as aromatic compounds in the environment have been shown to exhibit variable levels of cytotoxicity and mutagenicity in bacterial and mammalian cell systems. Occurrence of such chemicals with living organisms can have deleterious effects, often causing severe toxic as well as carcinogenic effects. Advances in the chemical industry during the past decades have greatly increased the number of man-made chemicals such as pharmaceuticals, pesticides, insecticides and other environmental pollutants, which in turn pose an ever increasing challenge to living organisms. To limit the threat posed by endogenously produced toxic compounds as well as the man-made chemical species, organisms have evolved complex detoxication systems fundamental to their survival.

DNT and TNT are nitroaromatic compounds that derive from ammunition. The release of these nitro-substituted explosives in the soil and surface waters due to military and industrial activities generates significant health risks to humans as well as to the ecological system. Due to their chemically resistant structures, a number of nitroaromatic compounds including DNT and TNT are highly recalcitrant to degradation and are mineralized at a slow pace by microorganisms. TNT is relatively more refractory to biodegradation than mono- and dinitrotoluenes due to its symmetric distribution of the nitro groups on the aromatic ring, limiting the attack of microbial enzymes that are fundamental in the biodegradation of aromatic compounds in the environment. TNT is considered a C-class human carcinogen and its metabolites have been shown to exhibit variable levels of mutagenicity in bacterial and mammalian cell systems. Occupational exposure of TNT has also been associated with various clinical manifestations including aplastic anaemia, dermatitis and toxic hepatitis. For the above stated reasons, the biotransformation of TNT has been extensively studied worldwide, and various enzymes from plants and microorganisms have been reported to be involved in the metabolism of TNT. A recent study of Arabidopsis thaliana clarified that monodehydroascorbate reductase 6 plays a pivotal role in TNT toxicity by generating a nitrorous that promotes formation of reactive oxygen species. However, detoxication of TNT and related compounds in mammals and insects remains largely unexplored.

GSTs constitute large group of phase II detoxication enzymes evolved via multiple gene duplications to diverse classes of enzymes that fulfill a broad spectrum of functional roles including detoxication of xenobiotics. GSTs act by catalyzing the reaction of the tripeptide GSH with numerous different chemical structures, suggesting that GSTs might play an important role in the biotransformation of TNT and its metabolites. Previously, a few tau class GSTs from poplar and Arabidopsis have been shown to have catalytic activities with TNT. Two of the poplar GSTs were reported to be up-regulated in response to TNT. However, the observed catalytic activities of these tau class GSTs appears not to be efficient enough to play an important role in the biotransformation of TNT.

In order to identify catalytically efficient GST enzymes that could efficiently catalyze the biotransformation of DNT and TNT, we have subjected a set of human GSTs along with two epsilon class GSTs from D. melanogaster to activity studies. The results summarized in Table 1 demonstrate that DmGSTE6 and DmGSTE7 have higher catalytic activities with both substrates compared to the human GSTs and the plant enzymes previously studied. Among the tested enzymes, DmGSTE6 was characterized as the most efficient enzyme with a specific activity of 62.7 ± 2.6 nmol min⁻¹ mg⁻¹, which is > 1000 times more active than the previously reported poplar GSTU16 and GSTU45. DmGSTE7 was the second best enzyme with a specific activity of 20.0 ± 2.0 nmol min⁻¹ mg⁻¹, 3-fold lower than that of DmGSTE6. The catalytic activities of DmGSTE6 and DmGSTE7 with DNT as substrate were somewhat lower and of similar magnitude.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DmGSTE6</th>
<th>DmGSTE7</th>
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<tbody>
<tr>
<td>TNT</td>
<td>0.30 ± 0.06</td>
<td>0.91 ± 0.15</td>
</tr>
<tr>
<td>kₐ (s⁻¹)</td>
<td>0.05 ± 0.005</td>
<td>0.06 ± 0.008</td>
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<tr>
<td>k₉/K₉ (mM⁻¹ s⁻¹)</td>
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<td>0.062 ± 0.014</td>
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<tr>
<td>DNT</td>
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<td>17.78 ± 3.51</td>
</tr>
<tr>
<td>kₐ (s⁻¹)</td>
<td>0.74 ± 0.29</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>k₉/K₉ (mM⁻¹ s⁻¹)</td>
<td>0.018 ± 0.001</td>
<td>0.019 ± 0.005</td>
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4. Discussion

Living organisms are exposed to a large number of non-nutritional chemical species, referred to as xenobiotics. The interaction of such chemicals with living organisms can have deleterious effects, often causing severe toxic as well as carcinogenic effects. Advances in the chemical industry during the past decades have greatly increased the number of man-made chemicals such as pharmaceuticals, pesticides, insecticides and other environmental pollutants, which in turn pose an ever increasing challenge to living organisms. To limit the threat posed by endogenously produced toxic compounds as well as the man-made chemical species, organisms have evolved complex detoxication systems fundamental to their survival.

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Crystal structures of DmGSTE6 and DmGSTE7 have recently been determined. Both enzymes belong to the epsilon class of GSTs, which is not represented in mammals or plants, but the structures do not reveal any features that could explain the high TNT and DNT activities. The chain-folds of soluble mammalian, plant, and insect GSTs are highly similar and the CDNB activities of DmGSTE6 and DmGSTE7 are similar in magnitude to most mammalian GSTs. Therefore, the exact topography of the active site accommodating the electrophilic substrate obviously governs the substrate selectivity of the different enzymes.

In conclusion, the identification of two Drosophila enzymes with distinguishing high catalytic activities with TNT and DNT underlie the potential of genetic engineering of GSTs for applications in biotransformation and phyto remediation, as evidenced by the overexpression of GSTs in A. thaliana.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.12.003.

References