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

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ABSTRACT

The nitroaromatic explosive 2,4,6-trinitrotoluene (TNT) and the related 2,4-dinitrotoluene (DNT) are toxic environmental pollutants. The biotransformation and detoxication of these persistent compounds in higher organisms are of great significance from a health perspective as well as for the biotechnological challenge of bioremediation of contaminated soil. We demonstrate that different human glutathione transferases (GSTs) and GSTs from the fruit fly *Drosophila melanogaster* are catalysts of the biotransformation of TNT and DNT. The human GSTs had significant but modest catalytic activities with both DNT and TNT. However, *D. melanogaster* GSTE6 and GSTE7 displayed outstanding high activities with both substrates.

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

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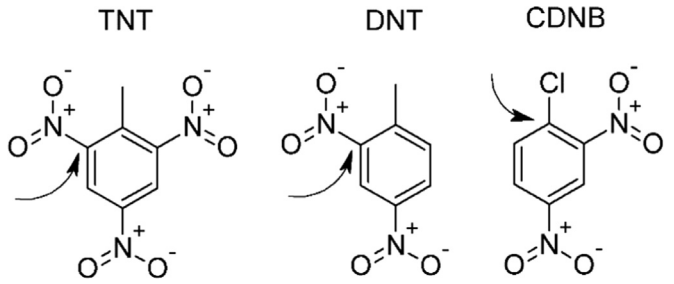

Fig. 1. Structural similarities between CDNB, DNT and TNT. The arrow head shows the site of attack by GSH.

Sigma-Aldrich. TNT was kindly provided by Dr. Rune Berglind, Swedish Defence Research Agency (FOI).

2.2. Expression and purification of recombinant GSTs

The genes encoding DmGST6, DmGSTE7 and human GSTS1-1 were custom synthesized by DNA 2.0 (Menlo Park, CA, USA) and were provided in the pJexpress 401 expression vector with N-terminal His_6-tags. *Escherichia coli* XL1-Blue electrocompetent cells were transformed by the electroporation technique and the bacteria were grown overnight on LB-agar plates containing 50 μg/ml kanamycin. A starter culture of 50 ml LB-medium containing the appropriate antibiotic was inoculated with a single colony and the cells were allowed to grow at 37 °C at 200 rpm in an incubator. After 16 h a larger culture of 500 ml LB-medium was inoculated with 5 ml of starter culture. The GST expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at an optical density of $OD_{600\ nm} \approx 0.4$. The bacteria were further allowed to grow at 37 °C for 16 h and cell pellets were obtained by centrifugation at 7000 rpm for 10 min at 4 °C. The supernatant was discarded and the 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% NaN_3) 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 27,200*g* 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 H_2O 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 H_2O), 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 DNT 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 k_{cat} and k_{cat}/K_m 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 K_m 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