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Crystal structure of the delta-class glutathione transferase in *Musca domestica*

Masayuki Sue [a, *], Shunsuke Yajima [b]

[a] Department of Agricultural Chemistry, Tokyo University of Agriculture, Sakuragaoka 1-1-1, Setagaya, Tokyo, 156-8502, Japan
[b] Department of Bioscience, Tokyo University of Agriculture, Sakuragaoka 1-1-1, Setagaya, Tokyo, 156-8502, Japan

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ABSTRACT

Among the various glutathione transferase (GST) isozymes in insects, the delta- and epsilon-class GSTs fulfill critical functions during the detoxification of insecticides. We crystalized MdGSTD1, the major delta-class GST isozyme in the housefly (*Musca domestica*), in complex with glutathione (GSH) and solved its structure at a resolution of 1.4 Å. The overall folding of MdGSTD1 resembled other known delta-class GSTs. Its substrate binding pocket was exposed to solvent and considerably more open than in the epsilon-class GST from *M. domestica* (MdGSTE2). However, their C-terminal structures differed the most because of the different lengths of the C-terminal regions. Although this region does not seem to directly interact with substrates, its deletion reduced the enzymatic activity by more than 70%, indicating a function in maintaining the proper conformation of the binding pocket. Binding of GSH to the GSH-binding region of MdGSTD1 results in a rigid conformation of this region. Although MdGSTD1 has a higher affinity for GSH than the epsilon class enzymes, the thiol group of the GSH molecule was not close enough to serine residue 9 to form a hydrogen-bond with this residue, which is predicted to act as the catalytic center for thiol group deprotonation in GSH.

1. Introduction

Glutathione transferases (GST) are one of the most prevalent enzymes. Cytosolic GSTs typically conjugate glutathione (GSH) to electrophilic substances, which become more hydrophilic and excretable. They are also involved in various other cellular processes that are unrelated to detoxification, such as isomerization of steroid hormones [1] or removal of reactive oxygen species. Because GSTs are involved in a wide variety of biological functions, their amino acid sequences feature a high diversity, which is used to divide GSTs into multiple classes. Although cytosolic GST classes have a low inter-class sequence similarity, they all share a canonical fold. Monomers are divided into two domains, the N- and C-terminal domains. The N-terminal domain adopts the thioredoxin fold, whereas the C-terminal domain consists of α-helices [2,3]. The substrate binding site is formed at the inter-domain cleft, in which a reduced GSH is bound to the GSH-binding site (G-site) at the N-terminal domain, whereas the second substrate, the electrophile, is retained by the hydrophobic binding site (H-site) at the C-terminal domain. Therefore, the sequence diversity is higher in the C-terminal domain than in the N-terminal domain, because of the structural variety of the electrophilic substrates.

Among the GST classes, the delta- and epsilon-classes are unique to insects [4,5]. They are known to play a crucial role in the emergence of resistance against insecticides including organophosphates, organochlorines, and pyrethroid [4,6–8]. In the housefly (*Musca domestica*), several delta-class (MdGST1 – MdGST4) and two epsilon-class (MdGST6A and MdGST6B) GSTs have been identified. First, Fournier et al. [9] showed that MdGST1 detoxifies organophosphorus insecticides. Then, a member of the epsilon-class GST, GST6A, was shown to have a greater activity toward organophosphates than delta-class GSTs [10]. (In the subsequent part of this paper, we refer MdGST1 and MdGST6A as MdGSTD1 and MdGSTE1, respectively, according to the nomenclature system of GSTs.) Hence, comparing the structures of both classes will contribute to knowledge about the underlying molecular mechanisms for substrate recognition and the emergence of insecticide resistance. The structural analyses of the delta- and

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; G-site, GSH-binding site; GSH, glutathione; GST, glutathione transferase; H-site, hydrophobic binding site.

* Corresponding author.
E-mail address: sue@nodai.ac.jp (M. Sue).

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epsilon classes have been mostly performed on *Anopheles* species and *Drosophila melanogaster* [7,11–13]. The epsilon-class GSTs have longer amino acid sequences than the delta-class enzymes because of their extended C-termini, which may affect their substrate specificity. However, there is no unequivocal evidence for a link between the C-terminal amino acid sequences and their substrate specificity. In our previous study, we solved the crystal structure of an epsilon-class GST, MdGSTE2 (an isozyme of MdGSTE1 that was formerly referred as MdGST6B) and analyzed its mode of the GSH binding in the catalytic site [14], which was the first report of a GST structure from *Musca domestica*. In this study, we analyzed the structure of MdGSTD1, one of the most abundant GSTs in *M. domestica*, and compared it with MdGSTE2. The two GSTs share about 40% amino acid sequence similarity. The C-terminal region in MdGSTD1 is shorter than in MdGSTE2, which could affect the architecture of the substrate binding pocket [7,14]. The aim of this study was to further characterization of insect GST structures by performing a crystallographic analysis of a delta-class GST, MdGSTD1.

2. Materials and methods

2.1. Cloning and expression of MdGSTD1

MdGSTD1 (GenBank accession no.: X61302) was cloned from the abdomen of the organophosphorus-resistant *M. domestica* strain Yachiyo that was maintained in our laboratory. MdGSTD1 cDNA was cloned by reverse transcription using Superscript III (Invitrogen) with an oligo (dT) primer followed by PCR using PrimeStar HS DNA polymerase (Takara Bio) with gene-specific primers. The amplified DNA was ligated into the *Nde*I-*Hin*dIII site of pET21a (Novagen), and then transferred into *E. coli* BL21 CodonPlus (DE3)-RIL. Expression and purification of the enzymes were performed as described previously [14].

2.2. Construction of MdGST6B mutants with deleted C-terminus

MdGSTE2 DNA fragments for generating C-terminal 6- and 12-residue deletions (MdGSTE2-Δ6 and MdGSTE2-Δ12, respectively) were amplified by PCR using the MdGSTE2 gene cloned in pET21a [14] as a template. The primer sets used for PCR were as follows (restriction sites are underlined): forward primer for both mutants, 5′-ACGCATATGGGAAAACTTGTTTTATATGGCAT; reverse primer for MdGSTE2-Δ6, 5′-AGAAAGCCTCTACTTCGATTTCACCATGGCCA, and reverse primer for MdGSTE2-Δ12, 5′-AGAAAGCCTCTACACTAATTGCTTAGCACCTG. The reaction was carried out using PrimeStar Max DNA polymerase (Takara Bio) with denaturation at 98 °C, annealing at 60 °C, and polymerization at 68 °C. Amplified DNA fragments were purified by agarose-gel electrophoresis and digested by *Nde*I and *Hin*dIII. The product was ligated to the *Nde*I–*Hin*dIII sites of pET21a and then transferred into DH5α. After sequence verification, the plasmid was introduced into BL21 CodonPlus (DE3)-RIL for heterologous expression of the enzyme. The purification protocol was same as described for the wild type.

2.3. Enzyme assay

The enzymatic activity of GST was measured spectrophotometrically according to the method of Habig et al. [15]. The K_m and k_{cat} values for 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) were determined by varying its concentration at a concentration of 1 mM GSH. The data were fit to the Michaelis-Menten equation using SigmaPlot (SPSS).

2.4. Crystallization and structure determination

For crystallization, 5 mg/mL of the purified MdGSTD1 in 10 mM Tris-HCl (pH 8.0) and 10 mM GSH were mixed at a 1:1 ratio with a reservoir solution containing 0.2 M sodium citrate, pH 5.6, 1.8 M ammonium sulfate, and 0.1 M tartrate. Crystals were obtained using the hanging drop vapor diffusion method at 20 °C, which grew crystals up to 0.3 x 0.3 × 0.2 mm in size within a few days.

Diffraction data for MdGSTD1 were collected using beamline 17A at the Photon Factory. Crystals were quickly soaked in a mixture of reservoir solution and 7 M sodium formate at a 1:1 ratio and flash frozen in a cold stream of nitrogen gas at 100 K. The datasets were merged and processed using the programs MOSFLM [16] and SCALA [17], respectively, in the CCP4 suite [18]. The initial structure of MdGSTD1 was solved by molecular replacement using the program MOLREP [19] in the CCP4 suite based on the coordinates of a target model, the mosquito GST (PDB code: 1JLV). Initial model building was performed by the program ARP/wARP [20], and iterative refinement and manual model building for the structures were performed with the program REFMAC5 [21] in the CCP4 suite and Coot, respectively.

3. Results and discussion

The k_{cat} values of MdGSTD1 to GSH and CDNB were 24.0 and 53.7 s^{-1}, respectively, and the K_m were 53.0 and 262 μM, respectively. Because of the low solubility of DCNB, it was not possible to precisely determine the kinetic parameters ($K_m > 800$ μM; $v = 0.1\ s^{-1}$ at 800 μM of DCNB). Interestingly, MdGSTD1 had a much higher activity for GSH ($k_{cat}/K_m = 0.453\ s^{-1}\ \mu M^{-1}$) than MdGSTE2 (less than $k_{cat}/K_m = 0.0047\ s^{-1}\ \mu M^{-1}$) [14]. However, the k_{cat} and K_m for CDNB for MdGSTD1 were about 5-times and 40-times higher,

Table 1
Data collection and refinement statistics.

	MdGSTD1 (PDB ID: 5ZWP)
Data collection	
Beamline	PF-17A
Wavelength (Å)	1.0000
Space group	$P2_1$
Cell Unit (Å, °)	$a = 51.3$, $b = 90.9$, $c = 51.4$, $\beta = 107.6$
Number of subunits per ASU	2
Resolution range (Å)	49.0–1.4 (1.48–1.40)
Completeness (%)	95.9 (95.9)
I/σ (I)	8.2 (5.9)
Redundancy	1.9 (1.9)
R_{merge}[a]	0.045 (0.115)
Number of unique reflections	84246 (11834)
Refinement	
Resolution (Å)	49.0–1.4 (1.436–1.4)
Number of reflections	84218 (5869)
R/R_{free}[b]	0.157/0.176 (0.184/0.212)
Number of atoms	
Protein	3364
Water	486
GSH	40
Formate	33
RMSD from ideality	
Bond length (Å)	0.015
Bond angle (°)	1.910
Average B factor (Å^2)	
Protein	8.73
Water	20.1
GSH	8.59
Formate	21.6

The numbers in parentheses are given for the highest resolution shells.
[a] $R_{merge} = \Sigma_h\Sigma_i|I_i(h) - \langle I(h)\rangle|\Sigma_h\Sigma_i I_i(h)$, where $I_i(h)$ is the ith measurement.
[b] A subset of the data (5%) was excluded from the refinement and used to calculate R_{free}.

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