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Note

Investigation of the active site of an unclassified glutathione transferase in *Bombyx mori* by alanine scanning

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Glutathione transferase (GST) is an important class of detoxification enzymes that are vital for defense against various xenobiotics and cellular oxidative stress. Previously, we had reported an unclassified glutathione transferase 2 in *Bombyx mori* (bmGSTu2) to be responsible for detoxifying diazinon. In this study, we aimed to identify the amino acid residues that constitute a hydrogen-bonding network important for GST activity. Site-directed mutagenesis of bmGSTu2 suggested that residues Asn102, Pro162, and Ser166 contribute to its catalytic activity.

Keywords: glutathione, glutathione transferase, Lepidoptera, site-directed mutagenesis.

Introduction

Glutathione transferases (GSTs, EC 2.5.1.18) are ubiquitously expressed and are responsible for intracellular detoxification of various xenobiotic and endogenous substances by catalyzing conjugation with glutathione (GSH).[1,2] Insect GSTs and their role in insecticide metabolism have been of particular research interest. Various GST classes (delta, omega, sigma, and zeta), as well as unclassified GSTs, have been characterized in *Bombyx mori*.[3–10] Recently, X-ray structures of delta-class (bmGSTD), sigma-class (bmGSTS), omega-class (bmGSTO), unclassified (bmGSTu), and unclassified 2 (bmGSTu2) GSTs in *B. mori* have been determined.[5,6,8,9,11] We have reported bmGSTu2 (Protein Data Bank (PDB) ID: 5ZFG) to catalyze diazinon metabolism, thus explaining its role in the insecticide resistance of *B. mori*. To improve our understanding of the molecular basis of bmGSTu2 catalysis, we have examined the structure and catalytic function of bmGSTu2. Silkworms provide a suitable model for studying lepidopterans; therefore, comprehensive studies on GSTs in *B. mori* could provide better insight into ways of combatting species that are considered agricultural pests.

Materials and Methods

1. Protein preparation

Recombinant bmGSTu2 was overexpressed and purified according to previously published methods.[12] bmGSTu2 mutants were constructed with a plasmid containing the coding sequences of wild-type bmGSTu2 and amino acid substitutions using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) in accordance with the manufacturer's recommendations. The nucleotide sequence of full-length mutant cDNA was checked by DNA sequencing.

2. Enzymatic activity

GST activity was measured spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) and 5 mM GSH as standard substrates. Each sample was measured three times (number of occurrences (n): 3), and each independent experiment was repeated three times (number of repetitions (N): 3). Data from assay conditions, under varying substrate concentrations in the presence of 5 mM GSH, underwent nonlinear regression analysis using the least squares method on KaleidaGraph software (HULINKS Inc., Tokyo, Japan), to determine the kinetic parameters, K_m and k_{cat}.

Results and Discussion

1. Amino acid residues of bmGSTu2 interacting with GSH

A phylogenetic tree showed bmGSTu2 to be close to delta- and epsilon-class GST, which are insect-specific GSTs.[16] Previously, we had identified the amino acid residues (Ile54, Glu66, Ser67, and Asn68) for the GSH-binding site using modeled bmGSTu2 and adGSTd1-6 (PDB ID: 1PN9).[16] The structure of apo-bmGSTu2 was also determined by X-ray crystallography.[10] Since the crystal structure of bmGSTu2 is the apo form, amino acid residues interacting with GSH could be identified by superimposition with GST structures including GSH followed by alanine scanning. Comparing the structures of bmGSTu2 (PDB ID: 5ZFG) and adGSTd1-6 (root mean square deviation (RMSD) = 1.4 Å) revealed that the GSH-binding site of bmGSTu2 was unchanged.

2. Hydrogen-bonding network

A hydrogen-bonding network is considered essential for GSH ionization, which, in turn, is required for catalysis.[17–19] The active sites of insect GSTs and the hydrogen-bonding network have been well characterized in *Anopheles dirus* GST D3-3 (adGSTD3-3).[20] Configuration of the glutamyl α-carboxyl group

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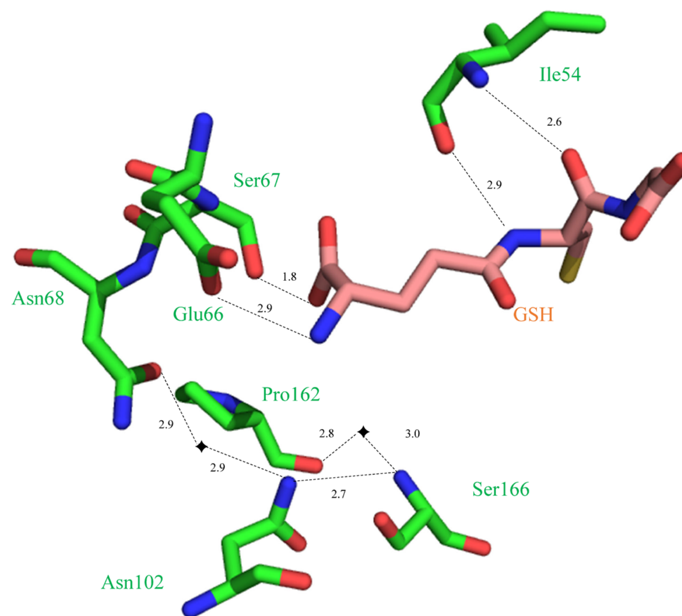

Fig. 1. Amino acid residues in bmGSTu2 proposed to be interacting with GSH. Carbon atoms of bmGSTu2 and GSH are in green and magenta, respectively. Other atoms, such as oxygen, nitrogen, and sulfur, are represented in red, blue, and yellow, respectively. Names of amino acid residues for bmGSTu2 and GSH are shown in green and magenta, respectively. Dotted lines and black stars represent hydrogen bond and water molecules, respectively. The length of each hydrogen bond is written beside the corresponding bond.

of GSH, together with the GSH binding-site residues Ser65, Arg66, Asp100, Thr158, and Thr162 of adGSTD3-3, facilitates the formation of a hydrogen-bonding network for the distribution of charge in terms of either a proton or an electron. Ionic interactions between the GSH glutamyl α-amino group and the carboxylic group of Glu64, as well as between Arg66 and Asp100, occur in the hydrogen-bonding motif. We had previously revealed that these residues correspond with Ser67, Asn68, Asn102, Pro162, and Ser166 in bmGSTu2.[5] Among the five residues, we had characterized Glu66, Ser67, and Asn68 in our previous study.[12]

In the structure of agGSTe2 (*Anopheles gambiae* epsilon-class GST) as well, the hydrogen-bonding network had been proposed. Superimposition of the structures of bmGSTu2 and agGSTe2 (PDB ID: 2IMI) revealed that Lys52, Ile54, Glu66, Ser67, and Ser111 correspond with the hydrogen-bonding network (His53, Ile55, Glu67, Ser68, and Arg112, respectively) in agGSTe2 (Fig. 1). Due to the long distance between the side chains of Lys52 and Ser111 in bmGSTu2 and GSH, the hydrogen bond has difficulty forming. Among the five residues, Ile54, Glu66, and Ser67 have already been characterized as glutathione-binding sites.[10]

Based on two sets of comparisons between the bmGSTu2 structure and adGSTD3-3 and agGSTe2, we proposed the hydrogen-bonding network for bmGSTu2 as shown in Fig. 1.

3. *Exploration of the bmGSTu2 active site by alanine scanning*

The superimposed structure of bmGSTu2 and agGSTd1-6 indicated the possibility that Ile54, Glu66, Ser67, and Asn68 residues of bmGSTu2 belong to the GSH-binding site.[12] As for the other amino acid residues in Fig. 1, we have not characterized Asn102, Pro162, or Ser166 in bmGSTu2. To determine which of these could contribute to the catalytic activity of bmGSTu2, all three residues were converted to Ala using site-directed mutagenesis. The resulting mutants were called N102A, P162A, and S166A, respectively. After purification from *E. coli*, each preparation migrated as a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The kinetic parameters of the mutants were compared with those of the wild-type enzyme using CDNB and GSH (Table 1).

Table 1. Comparison of kinetic parameters in bmGSTu2 mutants[a)]

Substrate	CDNB			GSH		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Wild	0.28	9.4	34	0.89	5.1	5.7
N102A	0.94	2.1	2.2	5.3	1.2	0.23
P162A	2.1	10	4.8	6.8	4.2	0.62
S166A	0.72	6.8	9.4	1.8	5.4	3.0

[a)] Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione. Data exhibits the averages from three independent experiments.

Interestingly, catalytic efficiency (k_{cat}/K_m) was reduced in all of the mutants. The most notable change was the N102A mutant's loss of activity. Asn102 in bmGSTu2 corresponded with Asp100 in adGSTD3-3. The phylogenetic tree revealed that bmGSTu2 is close to delta- and epsilon-class GSTs (Yammaoto *et al.*, 2018). The RMSD value between structures of bmGSTu2 and adGSTD3-3 (PDB ID: 1JLV) was 1.4. When Asp100 was replaced by Ala, the activity of adGSTD3-3 decreased. Since Asp100 interacted with Arg66 in adGSTD3-3, this mutation resulted in the disruption of the hydrogen network.[17] The catalytic efficiencies of N102A toward CDNB and GSH were 15 and 20 times lower than that of the wild type (Table 1), respectively. The Asn102 side chain could interact with the Ser166 side chain by a hydrogen bond. The mutation of Ser166 decreased the catalytic efficiencies toward CDNB and GSH (Table 1). There could be hydrogen bonds *via* water molecules between the nitrogen atom of the Asn102 side chain and the oxygen atom of the Asn68 side chain. Our finding revealed the interaction of Asn102 with Asn68 *via* hydrogen bonding. Asn68 of bmGSTu2 had been characterized previously by alanine scanning, and the catalytic efficiency was found to have decreased.[12] Collectively, these kinetic studies demonstrated that the mutation of residues in a hydrogen-bonding network results in decreased enzyme activity.

DmGSTE6 structure (PDB ID: 4pnf) has an RMSD value of 1.7 with that of bmGSTu2, revealing a novel epsilon clasp motif that is conserved in the insect order Diptera. This motif appears to contribute to the structural stability of the dimeric GST enzyme.[21] The motifs are composed of Ser68, His69, His101, Ser104, and Ser163 residues. The superposition of DmGSTE6 (PDB ID: 4YH2) with bmGSTu2 revealed that the corresponding motifs in bmGSTu2 were Ser67, Asn68, and Leu99. We had previously reported that Leu99 of bmGSTu2 forms part of the lock-and-key motif, which is crucial for stabilizing the hydrophobic interactions of GST monomers,[5] while Ser67 and Asn68 of bmGSTu2 are GSH-site residues.[12] We could not find any motif similar to the epsilon clasp motif in bmGSTu2.

Here, we converted Pro162 to Ala and demonstrated that the catalytic efficiencies toward CDNB and GSH were 8.6- and 3.6-fold lower than those of the wild type (Table 1). Pro162 does not have heteroatoms in its side chain, and the main chain interacted with the side chain of Ser166 *via* water molecules (Fig. 1). Since alanine scanning influenced the activity of bmGSTu2, the corresponding residue in bmGSTu2, namely Pro162, was considered to be involved in the structural stabilization of bmGSTu2. We measured diazinon-metabolizing activity using high-performance liquid chromatography. Since the activity of each bmGSTu2 mutant was not observed, they possibly could not metabolize diazinon significantly, suggesting that each amino acid is involved in substrate (specifically diazinon) recognition.

In conclusion, we have identified amino acid residues Asn102, Pro162, and Ser166 in bmGSTu2 as being responsible for its catalytic properties. Since the available crystal structure of bmGSTu2 is of apo-bmGSTu2, one GSH-complexed enzyme may provide more accurate information on the side-chain conformations of these residues. It would be interesting to study how CDNB or diazinon interacts with bmGSTu2 molecules; to this end, we are currently co-crystallizing the substrates. A structure-function study would make it possible to assist in the rational design of more effective pesticides.

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